

**APPLICATION  
OF SINGLE-MOLECULE SENSING  
FOR MEDICAL DIAGNOSTICS**

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**To my parents and my sister**

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# 1. Introduction

The search for molecular markers that predict the prognosis of individual patients or the response to a gene specific treatment is a major focus in medical research [1,2]. Patient progress can be assessed by detailed measurements of specific molecular indicators from bodily fluids or biopsies, such as RNA expression, protein expression, protein modification, or concentration of metabolites.

Herceptin<sup>®</sup> for blocking of Her2 receptor mediated tumor growth in metastatic breast cancer has become a paradigm for the feasibility of targeted therapy [3-5]. Glivec<sup>®</sup>, targeting the Kit-receptor is effective in both chronic myeloid leukemia [6,7] and gastrointestinal stroma tumors [8], indicating that targeted therapy is not necessarily limited to a single cancer type. The identification of specific gene expression profiles that predict response to docetaxel (Taxotere<sup>®</sup>) treatment in breast cancer, or the finding that EGF receptor mutations are an indicator for response to gefitinib (ZD1839, Iressa<sup>®</sup>) treatment in lung cancer [9]. It can be expected that in the near future numerous additional molecular markers will be identified for a variety of different neoplasias. But the monitoring of molecular markers is not only of interest to cancer researchers and is certainly not limited to neoplasias.

Recent results in ophthalmological research for instance show that lymphocytes in the blood respond to glaucoma or glaucomatous damage with a clear difference in gene expression [10-12].

These developments prompt for the investigation of rapid, reliable, sensitive and cheap assays for the detection and quantification of therapy-relevant target genes for possible therapeutical applications. Diagnostic methods used to date, including the detection of DNA copy numbers (e.g. by fluorescence in situ hybridization [13], quantitative

PCR [14], Southern blotting), RNA expression (RT-PCR, RNA in situ hybridization, Northern blotting [15]) or protein levels (immunohistochemistry (IHC), Western blotting [16]), can hardly meet these criteria. Although being of great importance, such techniques share the disadvantage that they are time consuming, expensive and require extensive pre-treatment of samples.

An optimal diagnostic tool should allow the parallel investigation of multiple markers in situ, with only minimal tissue requirement but maximal sensitivity and specificity.

Ultrasensitive detection schemes based on fluorescence have seen a tremendous progress during the last ten years. Detection of single fluorescent molecules has become a standard tool in various fields of research ranging from biological physics over material science to quantum optics [17-25]. The ultimate sensitivity of detecting a single fluorescent molecule is due to the extreme specificity of fluorescence. The absorption cross section for fluorescence processes is 10 - 11 orders of magnitude larger than the cross sections of competing effects that also generate red-shifted light. This means nothing else but that in the ideal case it is possible to detect a single fluorescent molecule in the presence of  $10^{10}$  environmental molecules. Such numbers can be easily reached by sufficiently reducing the effective illuminated volume.

The goal of this thesis is to develop and test a simple, cheap and fast method that is able to quantify ultra-small concentrations of relevant molecular targets using an optical detection scheme based on single-molecule fluorescence. In order to keep the setup simple but still have a built in potential for parallelization and larger-scale integration and miniaturization, we decided to excite and detect single molecules through an optical waveguide. The use of an optical waveguide bears the tremendous advantage of being compatible with lab-on-the-chip platforms. Our approach provides the basis for the implementation of single-molecule detection assay in lab-on-the-chip architectures which

have the potential to completely outrange today's techniques in molecular diagnostics since they are more specific, sensitive, faster and cost effective.

The thesis is a compilation of 4 publications to be submitted set down as 5 subsequent chapters.

The outline is as follows:

The second chapter introduces the optical setup used and discusses its principles of operation. A first qualitative proof-of-principle of the detection principle is provided.

The third chapter deals with the problem of detecting transient signals, like fluorescence bursts, in the presence of significant background noise. While it is the common opinion that single molecules can only be detected under extreme low noise conditions, here we demonstrate that we can reliably count single fluorescence bursts in the presence of significant background noise accumulated in the optical waveguide.

The fourth chapter addresses the question of optimal operation conditions for the setup. All relevant parameters are discussed and their optimal values are determined in experiments.

The fifth chapter demonstrates that the setup may be used as an optical biosensor that is able to quantify the concentration of certain target molecules in a liquid. To this end we demonstrate a linear relationship between concentration and number of detected fluorescence bursts. A dynamic range of many orders of magnitude is demonstrated starting at pM concentrations going down to one aM.



The sixth chapter demonstrates that the optical setup can be used in combination with highly specific molecular beacons that are able to detect the presence of target mRNA sequences. The detection of complementary targets in buffer is demonstrated. For a fixed concentration of molecular beacons the concentration of targets can be monitored by determining the ratio of open to closed beacons.

Finally, after establishing this new single-molecule detection and quantification method including the use of molecular beacons the eighth chapter offers an outlook to future applications in blood. For this purpose a similar experiment as described in chapter six is performed, however by replacing the buffer solution with human blood. The results proof that the method at hand is also suitable to work in body fluids with residuals of corpuscular elements and that their autofluorescence properties do not interfere with the sensor's function.

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## **2. Novel method for the detection of fluorophores in liquids**

### ***2.1 Introduction***

The emphasis on new highly sensitive and specific biomarkers for the early detection of molecular caused diseases marks a current trend in the biomedical sector [1-8]. The rapid assessment of predictive factors that can also serve as targets for a therapeutical approach requires proficient investigation methods and tools, able to perform at the single-molecule level. The ability to detect for instance DNA or mRNA at the single-molecule level would render amplification techniques, such as polymerase chain reaction (PCR) [9], superfluous. It also would minimize the need for sample pretreatment and thus would allow for a more direct investigation of native material.

The detection and quantification of biomarkers is widely linked to the detection of fluorescence as seen in immunoassays, flow cytometry and chromatographic analysis. For these techniques the detection limits range between  $10^3$  and  $10^6$  fluorescent molecules [16], while automated DNA sequencing is limited to the range of  $10^6$  to  $10^7$  DNA molecules and additionally requires PCR [17,18]. Fluorescence however holds the potential for single-molecule detection in the attomolar range and even below [19-22].

During the past ten years, new fluorescence techniques have evolved capable of detecting single molecules in solutions [20,21]. Most of these methods however rely on the use of objectives [23,24]. The advantage of using integrated optics such as optical transducers instead of objectives would allow for miniaturization [14]. Unfortunately the current examples using waveguides are far from reaching single molecule

detection. With the here-presented method it can be shown that it is possible to detect single molecules fluorescence through a waveguide.

In the following chapter, we present a novel optical fluorescence detection technique that allows for remote single-molecule detection of fluorescent-labeled oligonucleotides in a liquid environment at room temperature. Remote sensing is achieved by detection through an optical waveguide, c.f. a glass fiber. Both, the excitation light and the fluorescence signal are coupled through an optical fiber thus implementing a remote detection scheme. The background luminescence created in the glass fiber by the strong excitation light can largely be suppressed by the use of a wavelength-shifting concept. The ability to detect free-floating molecules accentuates the potential of this method: Complicated chemical modification of surfaces can be avoided since no adsorption of molecules to any kind of sensor structure is required. We finally discuss the detection efficiency of the glass fiber by means of dipole radiation patterns near the glass/water interface.

## ***2.2 Materials and Methods***

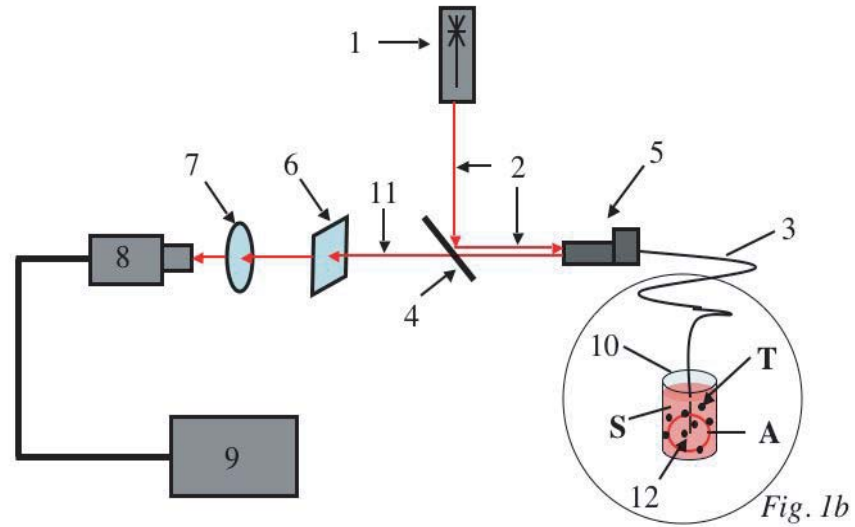
### **2.2.1 Experimental Setup**

A scheme of the setup is shown in Fig. 1. Excitation light **2** is provided by a He Ne laser (HeNe,  $\lambda=632.8$  nm, max power 35 mW) **1**. A fiber aligner **5** (Fiber Positioner Kit, FS/S, New Focus) is used to couple the light into a single-mode fiber **3** (ClearLite 630-11,#cf042447, length ca. 0.4 m, Laser Components). The fiber consists of a dielectric material of higher index than the test solution **S** which is the case for standard liquids and glass fibers. In the test solution, target molecules **T** of interest are excited by the light emitted at the vertically cleaved far fiber end causing the target molecules to emit fluorescence. The

fluorescence of molecules that are sufficiently close to the fiber is coupled back into the fiber and is emitted at the other end. Here it is collimated by a microscope objective included in the fiber aligner. The beam of fluorescence passes a dichroic mirror **4** (XF3307 800WB80 17311, Omega Optical Inc., AR Coat R 633) and an optical filter **6** (T740/140 650 dcip, cube 38x26, Chroma Technology Corp.). The latter filter cuts off the excitation light and passes the fluorescence. The fluorescence is then focused to the 200  $\mu\text{m}$  active area of a single-photon counting avalanche photodiode **8** (SPAD, Single Photon Counting Module; dark count rate < 250 c/s, SPCM-AQR-13, Perkin Elmer). The lens has a focal length of 200mm to ensure that the image of the fiber core on the SPAD is only slightly smaller than the SPAD's active area. This avoids the detection of excess auto-fluorescence from the fiber cladding. Finally, the SPAD is read out by a computer equipped with a counter/timer board **9** (Labview 7.1, BNC 2120, NI Multifunction Board, NI PCI-6052E I/O, Shielded Connector BLK, SCB-68 BLK, National Instruments).

A test solution containing target molecules of interest is prepared and presented in a self-designed PMMA fluid cell **10**, **Fig. 1 (b) IV** able to contain up to 1.5 ml of test solution. A mechanical stirring device **Fig. 1(b) I and III** ensures proper initial homogenization of the solution and is then able to rotate with up to 25000 rpm. When in use, the freshly cleaved (Miller Stripper Fo 103-S Oski, Fiber Cleaver S315, Furohawa, Mesomatic, Cham, Switzerland) end of the optical fiber **3**, **Fig. 1(b) II** is immersed about 1 cm in the solution **S**.

(a)



(b)

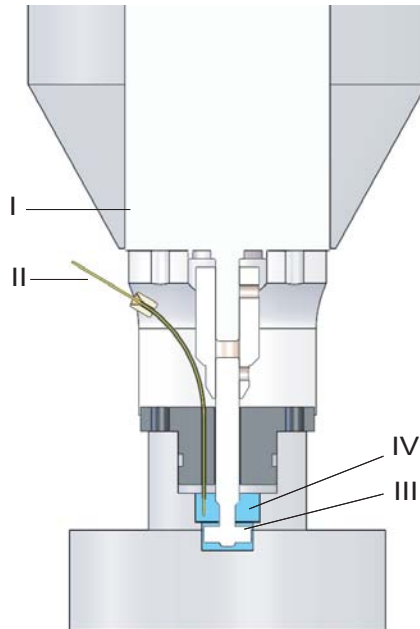


Figure 1(a): Experimental setup. A HeNe-Laser is coupled into a glass fiber via a dichroic mirror. At the end of the glass fiber, single fluorophore molecules are excited and couple a part of their fluorescent signal back into the fiber. The fluorescent signal passes the dichroic mirror, is filtered and is then focused onto a single photon counting module.

Fig. 1(b): Motor (I), glass fiber (II), stirrer (III), fluid chamber (IV).

### 2.2.2 Design of FRET molecules

In order to bypass the background noise of the optical setup that is mostly generated in the optical fiber [16], fluorescence resonance energy transfer (FRET) [17,18] is used to achieve a large effective Stokes shift of the fluorescence of the labelled target oligonucleotide sequence which consists of a quintuple thymine base sequence. The 5'-end fluorophore donor was Cy5.5 and the Cy7 fluorophore was used as the 3'-end acceptor. Cy5.5 and Cy7 belong to the class of cyanine dyes. All FRET target molecules were purchased from Genelink, Hawthorne, California, USA.

Cyanine dyes [19,20] are synthetic dyes containing a chain of  $(-CH=)_n$  groups forming a conjugated system linking two nitrogen-containing heterocyclic rings together.

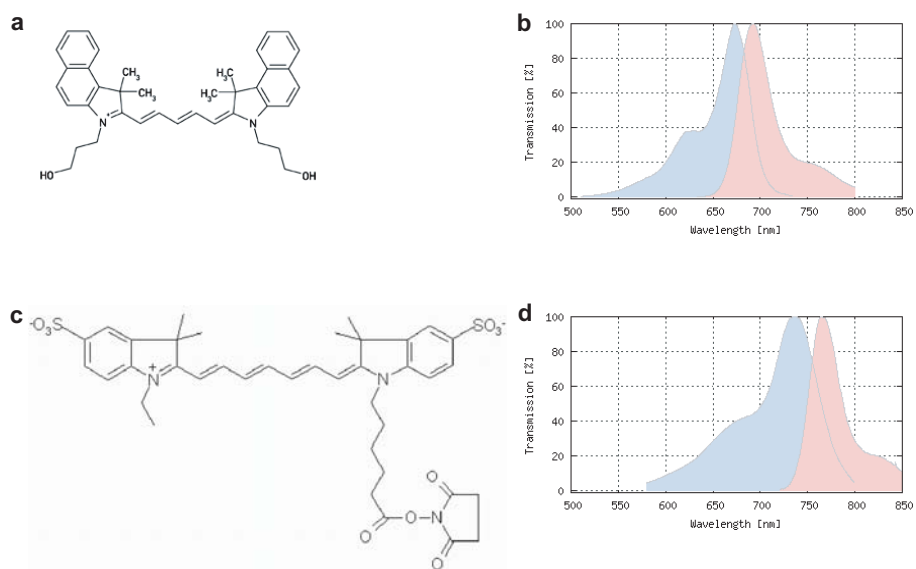


Figure 2. (a) Structure of Cy5.5. (b) Absorbance (blue curve) and emission (red curve) spectra of Cy5.5 (absorbance max. 675nm, emission max. 694nm). (c) Structure of Cy7. (d) Absorbance (blue curve) and emission (red curve) spectra of Cy7 (absorbance max. 743nm, emission max. 767nm).

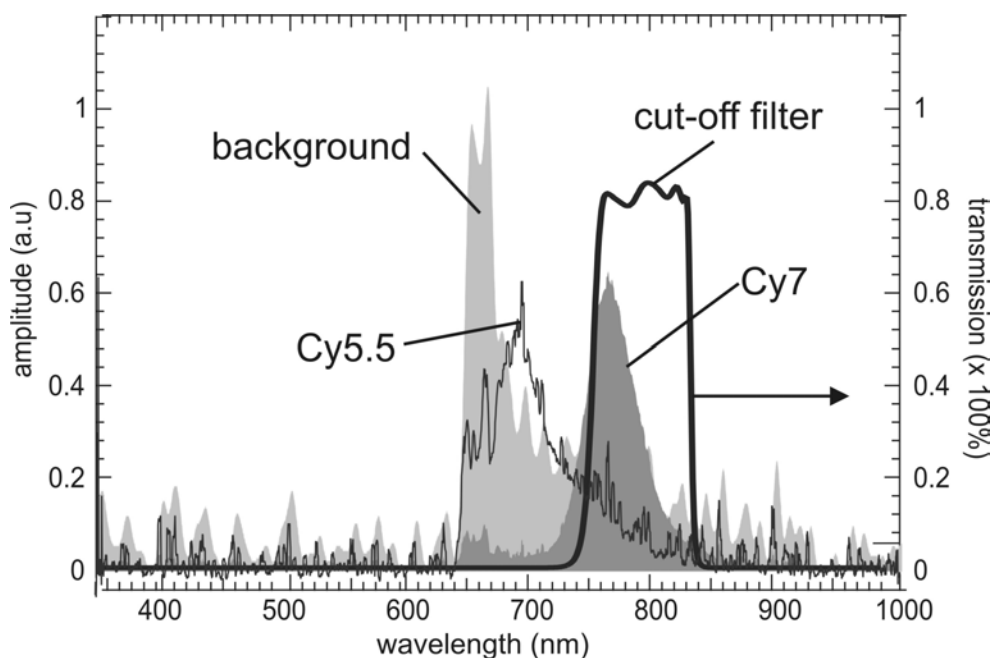
### 2.2.3 Wavelength shifting and filter design

Sending high-power excitation light over an optical fiber bears the disadvantage that inside the fiber background luminescence caused by various effects, like e.g. Raman scattering, is accumulated over the whole length of the fiber [13,14,21]. It has been found that indeed the intensity of the autofluorescence indeed scales linearly with the fiber length at a constant input power.

All spectral measurements were recorded with an USB2000 mobile spectrometer from Ocean Optics Inc. For that matter the SPAD (8) as seen in Figure 1 was replaced by the spectrometer. The cutoff filter (6) was removed and replaced by a holographic notch filter that cuts off the laser line. The excitation power for all fluorophore and background measurements was 2 mW. The integration time for all spectra was 5 sec. A stirring rate of 1000 rpm was chosen to avoid local bleaching. The concentration for both Cy5.5 and oligo FRETs was 50 nM each.

The spectrum of the background shows discrete lines indicative for a Raman process and falls off slowly towards longer wavelengths (see Fig. 3). For a fiber of a length of about 50 cm the amount of background luminescence in the relevant spectral window is so large, that detection of fluorescent molecules, like Cy5.5, excited at 632.8 nm, with a typical Stokes shift of about 50 to 60nm, is hardly possible since their emission spectrally overlaps with the background spectrum (see Fig. 3). In order to enable the detection of single fluorescent molecules a FRET pair consisting of a short oligonucleotide labelled with Cy5.5 as donor and Cy7 as acceptor is used. The FRET pair can be viewed as an effective chromophore that upon excitation at 632 nm emits fluorescence at 767 nm [22].





*Figure 3: Spectra of fluorophores and filters. Fluorescence spectra have been recorded with the cut-off filter replaced by the notch. Background is the luminescence background of the fiber dipped into a buffer solution. Cy5.5 is the fluorescence spectrum of the dye Cy5.5 plus fiber background. Cy7 is the spectrum of a FRET pair with Cy7 as acceptor and Cy5.5 as donor. Cut-off filter is the transmission characteristics of the cutoff filter used. The wavelength shifted emission of the FRET acceptor Cy7 is efficiently detected while cutting off a significant part of the fiber background.*

Using a cut-off filter with a bandpass centered at 795 nm it is possible to detect a good portion of the fluorescence while cutting off a significant part of the background. As will be shown in the following, this strategy allowed the detection of single FRET pairs through the optical fiber.

## 2.2.4 Recording of time traces

Data are recorded by counting the number of photons detected for series of subsequent time bins. The bin width was 100  $\mu$ s in all

experiments. The total duration of experiments was 1 min if not specified otherwise.

### **2.2.5 Buffer solution**

The oligonucleotides were diluted using a buffer solution containing 10mM Tris-HCl, pH8.3, 50mM KCl, 1.5mM  $\text{MgCl}_2$  and ultrapure RNase-free water (Sigma). All experiments were conducted at room temperature.

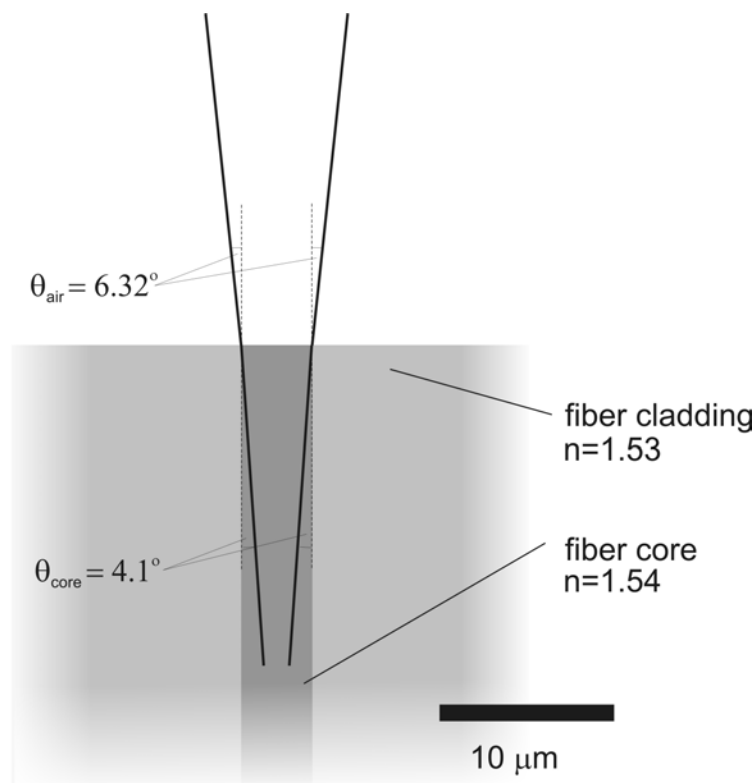
### **2.2.6 Data treatment**

An algorithm allowing to unambiguously detect transient burst-like signals in presence of stationary noise was programmed in Labview7.1. In order to discriminate a transient signal from the background noise an optimum threshold is determined using an iterative algorithm that isolates the probability distribution of the background noise. Knowledge of the probability distribution of the noise allows excluding the detection of false positive events with a defined probability by choosing a threshold such that for a signal consisting solely of background noise the probability for the detection of a noise peak above the threshold may be neglected. (See chapter 4).

### **2.2.7 Characteristics of the optical fiber**

Fig. 3 shows a sketch of the core area of a cleaved optical fiber. The numerical aperture (NA) of the fiber used in the experiments was  $\text{NA}=0.11$ . This corresponds to a full opening angle of the light cone emitted by the fiber into air of roughly  $12^\circ$ . This angle in turn also is the angle of acceptance for radiation to be coupled into a guided mode. Inside the fiber this corresponds to a propagation angle of up to  $4.1^\circ$  that

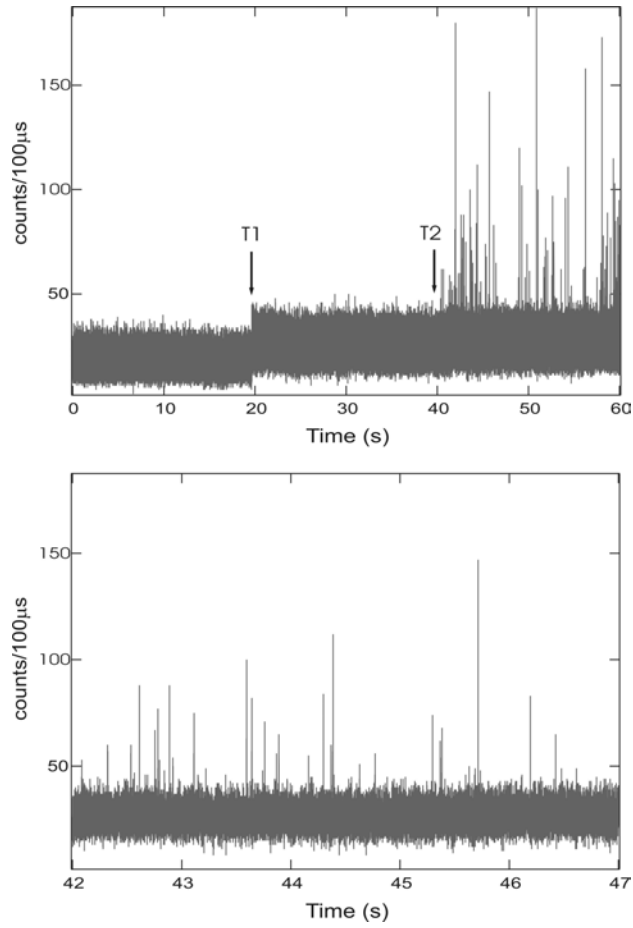
is accepted by the guided mode. The mode field diameter of the fiber is  $4.3\text{ }\mu\text{m}$ .



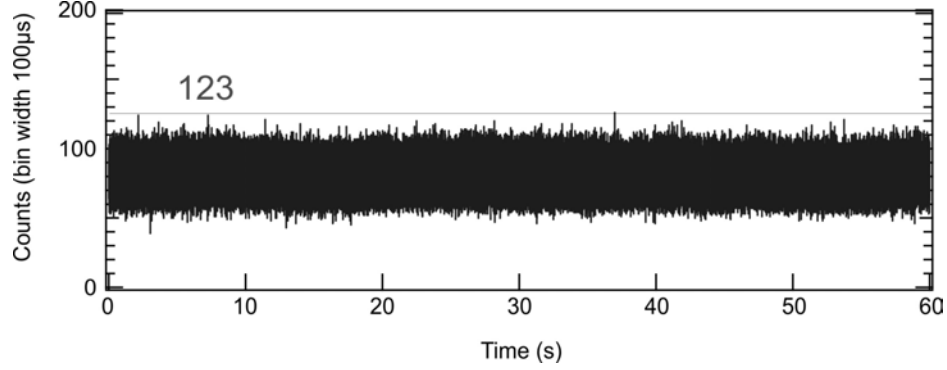
*Figure 4: Parameters of the optical fiber. Note that only the core region of the fiber is shown. The diameter of the fiber without plastic coating is  $125\mu\text{m}$ .*

## **2.3 Results and Discussion**

The setup of Fig. 1 was used to detect the presence of oligo FRETs in a buffer solution. The fiber was dipped into a test solution containing Cy5.5-Cy7 oligo-FRET molecules in a concentration of  $1\text{ nM}$ . Fig. 5 shows the result of such an experiment. The recording of data was started while the fiber was still in air.



*Fig. 5: Recording of fluorescence time traces. Upper panel: 60s experiment. The first 20s show signal measured without dipping the fiber into the test solution. At time T1 the fiber dips into the sample solution with a concentration of 1nM. At time T2 stirring (17000 rpm) is switched on and maintained. Lower panel: Zoom into the time trace showing individual fluorescence bursts. Excitation power: 1.8mW.*



*Fig. 6: Experiment in a buffer solution with optimal stirring. The line shows the threshold as determined by the burst detection algorithm (chapter 3).*

A stable background signal is recorded without any bursts, as expected. At time  $T_1=20\text{s}$ , the fiber is dipped into the test solution. Due to the comparatively high concentration of chromophores, the background signal increases slightly. This increase is the cumulative effect of a large number of fluorophores that are excited and couple back their fluorescence into the fiber core. At a concentration of  $1\text{nM}$  the average number of FRET pairs in a volume of  $1\text{ }\mu\text{m}^3$  is about 0.6. The diffusion constant of a larger molecule in water typically is  $D \approx 5 \cdot 10^{-10} \frac{\text{m}^2}{\text{s}}$  [25]

which means that according to  $\langle x^2 \rangle^{1/2} = \sqrt{2Dt}$  a distance of  $4\text{ }\mu\text{m}$ , which is the diameter of the fiber core, is covered in 16ms. If the true diffusion constant is five times smaller, than it already takes 80ms to cover  $4\text{ }\mu\text{m}$ . This is a too long time for efficient sampling of a larger sample volume using a fixed illuminated volume. Stirring is used to accelerate this process. At  $T_2=40\text{s}$  stirring (17000 rpm) is switched on. Due to the rapid flow of the liquid the appearance of fluorescence bursts is observed. The lower panel of Fig. 5 is a zoom of a fluorescence time trace showing fluorescence bursts above the background.

Fig. 6 for comparison shows the result of an experiment using a clean buffer solution. Although stirring is switched on, no fluorescence events can be detected.

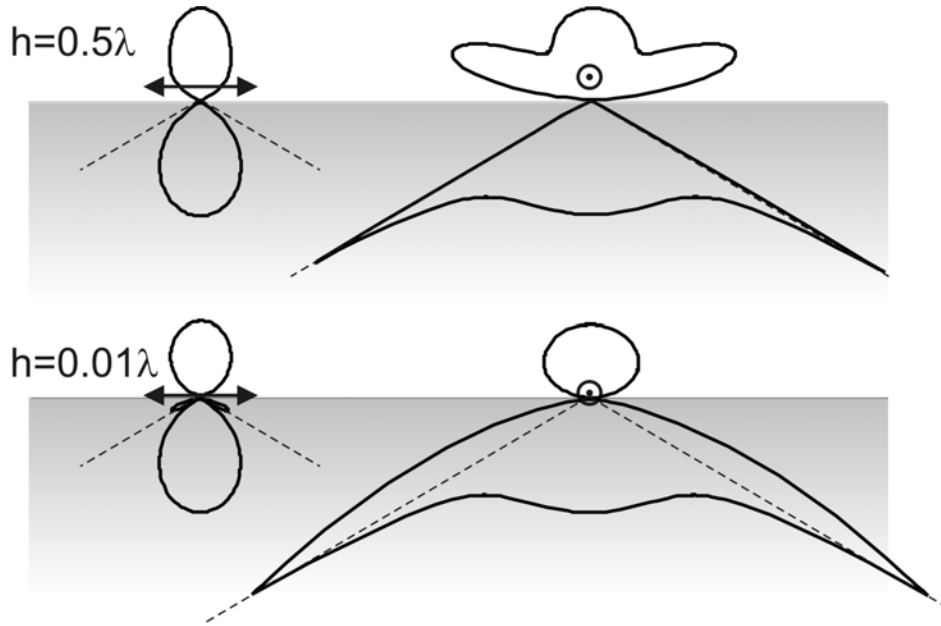
### 2.3.1 Simulations

The amplitude of the fluorescence bursts observed in Fig. 5 suggests that there is a significant efficiency for the collection of fluorescence by the fiber. To study this light collection process in more detail we consider the radiation patterns of single dipole emitters close to water/glass interface [13-15]. The refractive index of water is taken to be  $n_1 = 1.33$  – the refractive index of the guiding core is  $n_2 = 1.54$ . Once the radiation patterns are known, the collection efficiency can be determined by taking the ratio  $\eta$  of the total emitted power by the dipole in the full solid angle and the power emitted into the acceptance solid angle  $\varepsilon$  for guiding of light in the core.  $\Omega$  is the solid angle.

$$\eta = \frac{\int p(\Omega) d\Omega}{\int_{full} p(\Omega) d\Omega} \quad (1)$$

Here,  $d\Omega = \sin(\theta) d\theta d\phi$  if we assume spherical polar coordinates  $\theta$  and  $\phi$ . Since  $\theta$  is counted from the vertical axis, the integrands of (1) have a zero in the direction of the positive and negative z-axis thereby reducing the weight of the radiation in these directions.

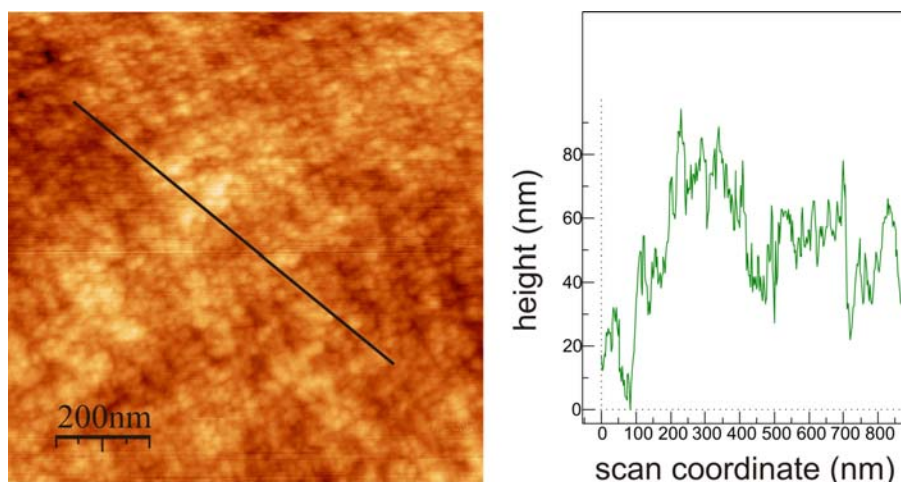
Fig. 7 shows emission patterns calculated for dipoles far away and in close proximity to the water/glass interface, respectively [15]. Most of the radiation is coupled into large angles close to the angle of total internal reflection (dashed line). Evaluating (1) for these patterns results in  $\eta = 0.1\%$  independent of the distance to the interface. This is because the emission pattern in the allowed zone for an infinitely extended interface does not change with distance. However, for the case of our fiber, we expect that only molecules with distances sufficiently small compared to the core diameter will significantly couple light back into the fiber.



*Fig. 7: Emission patterns of dye molecules close to a water glass interface, dipole parallel to interface. Upper panel: large distance, lower panel: close proximity. The dashed lines indicate the angle of total internal reflection. Note that the amount of light emitted into the allowed range is independent of the distance to the interface  $h$ . Left: Cut along the dipole. Right: Cut perpendicular to dipole orientation.*

The small detection efficiency raises the question how the large fluorescence bursts in Fig. 5 can be achieved by considering only a single molecule as a source. The bin width in the time traces of Fig. 4 is 100  $\mu$ s and the most probable burst amplitude is between 25 and 50 counts. Taking the collection efficiency of the fiber in account this corresponds to an emission rate of a detected molecule of between  $2.5 \cdot 10^8$  and  $5 \cdot 10^8$  photons/s. This corresponds to the saturation count rates of two-level systems with excited state lifetimes of between 4ns and 2ns which are compatible with the short excited state lifetimes of Cy dyes of around 1ns [15]. However, this also means that only molecules may be detected which have a nearly negligible triplet yield. In polymers, the triplet lifetimes have been shown to have a large spread which

suggests that a fraction of molecules with small triplet yields will exist [15]. The fast stirring that is necessary to observe significant numbers of peaks would be compatible with such an explanation since the exchange of material is fast enough to allow rather rare species to be detected at sufficient rates.



*Fig. 8: AFM image of the surface of a freshly cleaved fiber end face. Left: topography, right: Line profile along the black line in the topography image. Considerable roughness is observed.*

Another explanation for the high amplitude fluorescence bursts might be the fact that the glass/water interface represented by the cleaved fiber is not as smooth as assumed in theory. Fig. 8 shows an atomic force microscopy (AFM) image of an area of a freshly cleaved fiber in the vicinity of the fiber core. Considerable roughness is observed with excursions of up to 20 nm. The effect of such roughness on the emission patterns of single molecules that pass by in close proximity still needs to be investigated in detail using numerical methods.



## **2.4 Conclusions**

We successfully demonstrated the possibility to detect single fluorescent oligonucleotide molecules through a glass fiber in a liquid environment. The continuous monitoring of fluorescent signals as a function of time generates characteristic time traces which show fluorescence bursts which are identified with the signals of single or few fluorophores. The burst detection algorithm can discriminate the bursts from the strong Poissonian background generated mostly in the fiber. The method is capable of performing at room temperature in a conventional lab environment without any special requirements concerning light conditions or sterility. The optical information is gained instantaneously without having to revert to any kind of molecular adsorption procedures. The target molecules remain free-floating in solution. The buffer solution itself is simple to produce and requires no pretreatment whatsoever.

## **2.5 Outlook**

For the above-mentioned reasons the here presented novel detection method highly qualifies for applications within the biomedical sectors. Since the biosensor is able to perform with free-floating molecules, a combination with molecular switches, which alternate their fluorescent behavior upon binding to their specific targets, nearly imposes itself. A combination of this kind would allow for a broad spectrum of supplementary applications. Possible fields of interests would be e.g. the farming sector, varmint detection, materials research, e.g. investigation of repellent surfaces and of course the military sector, e.g. the detection of ultralow concentrations of bioagents.

The detection system with its high sensitivity also reserves the alternative of combination with microfluidic networks. Finally, the use of integrated optics would ensure the contingency for miniaturization and will lead to strongly enhanced detection efficiencies.

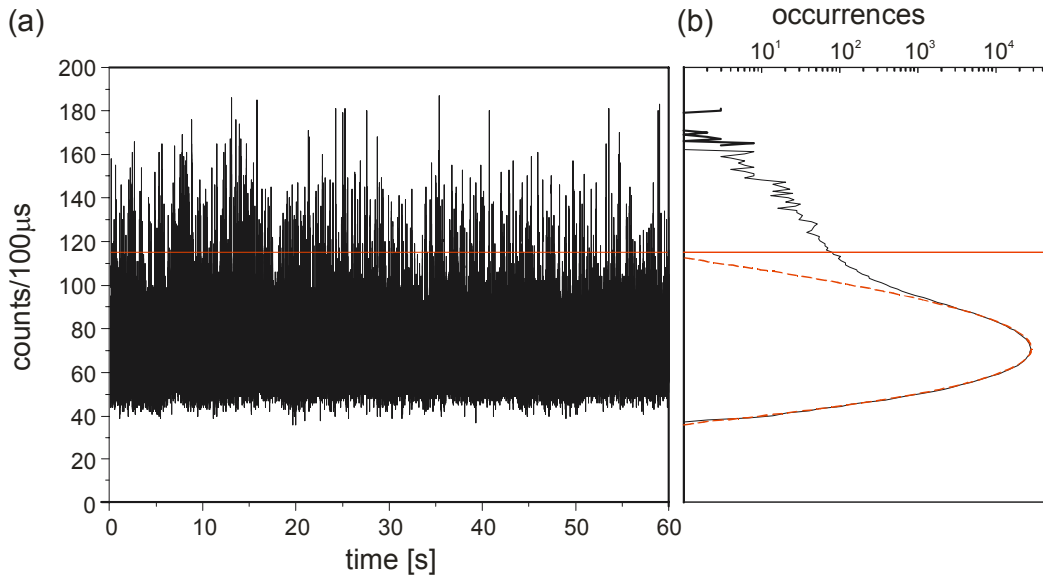
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### **3. Detection of transient events in the presence of background noise**

#### ***3.1 Introduction***

The detection of rare transient events (bursts) above a strong stationary background noise with a high level of confidence is a problem of broad interest in various sensing applications ranging from ultra-sensitive optical detection e.g. for biological assays or medical diagnostics, over electromagnetic sensors, to defence applications. In general, a transient signal is considered to be detected above the noise either if (i) its amplitude is many standard deviations above the mean value of the noise's probability distribution or if (ii) the wave form, i.e. the duration of the transient event is clearly distinct from the noise's characteristic fluctuations in time [1-4].



*Fig. 1. Time trace and histogram of a model data set. (a) Time trace with a bin width of  $100\ \mu\text{s}$  showing fluorescence bursts on top of a strong Poissonian background. (b) Histogram of the time trace in (a). The fluorescence bursts lead to a characteristic deviation from Poissonian statistics. The horizontal line shows the threshold level above which signals are counted as burst. The dashed curve plotted together with the histogram is the best estimate for the noise probability distribution obtained by calculating the mean of the noise after removing bursts above threshold (see text). A remarkably good agreement is obtained.*

Here we propose a method which is applicable in particular if the signal bursts are neither easily distinguishable from the characteristic fluctuations of the noise nor their amplitude is large enough to be considered clearly above the noise. The method is based on a fast converging iterative algorithm which determines an optimum threshold for the detection of bursts. It provides a quantitative measure for the probability of false positive events due to the background noise peaks which may be predefined by the user. The reliability of the method is assessed by performing Monte-Carlo simulations of the burst detection process. To demonstrate the method's potential we detect and count

single-molecule fluorescence bursts recorded in presence of a significant stationary background noise.

### 3.2 Algorithm

To simplify the discussion, but without loss of generality, we consider a data set describing a time series of counts per time interval containing rare transient events (bursts) in presence of a significant background noise with a Poissonian distribution. The algorithm outlined in the following can be easily adapted to accommodate different types of stationary noise, e.g. Gaussian noise. Apart from being sufficiently rare, no further assumptions are made with respect to the amplitude and shape distribution of the transient events superimposed to the noise. Fig. 1 (a) shows a time trace of a typical experimentally obtained data set that serves as an example along side with the respective histogram  $H(n)$  [Fig. 1 (b)]. Here  $n$  is the number of counts per 100  $\mu$ s. Fluorescence bursts of various amplitudes are observed above the background noise.  $H(n)$  shows a clearly distinguishable main Poissonian noise peak and a tail that accounts for the fluorescence bursts. As can be seen in Fig. 1 (b) the number of transient events characterized by the respective area of the histogram is small compared to the area of the noise peak. We note that  $H(n)$  may be thought of as consisting of a sum of two separate histograms - one describing the pure background noise and one describing the distribution of signal bursts such that  $H(n) = P(n) + S(n)$ , where  $P(n)$  describes the distribution of background counts and  $S(n)$  describes the distribution of signal burst heights. Signal bursts cannot be easily separated from the noise since both distributions overlap. To optimally discriminate signal bursts from similar events due to background noise a threshold must be determined above which a burst is counted as a signal burst. The threshold must on the one hand be low enough in order to miss as few as possible true signal peaks and

on the other hand it must be high enough to exclude the possibility to count a strong fluctuation of the noise as a signal. The latter would contribute to false positive events which in view of applications e.g. in medical diagnostics have to be minimized because of possible expensive consequences. To determine such an optimum threshold the probability distribution of the background,  $P(n)$ , in the present example the normalized Poissonian distribution characterized by its mean  $\mu$  and the variance  $\sigma = \sqrt{\mu}$

$$P(n) = \frac{e^{-\mu} \mu^n}{n!} \quad (1)$$

must be recovered from the data as precisely as possible. Assuming that this has been achieved, we may consider the probability distribution of the background alone. This enables us to determine a threshold for burst amplitude  $\chi$  by demanding that the absolute number of time intervals  $K$  for which the number of counts  $n$  exceeds the threshold  $\chi$  is smaller than a still tolerable small number, say e.g. 1.  $K(\chi)$  is determined as

$$K(\chi) = N \times \left( 1 - \int_{-\infty}^{\chi} P(n) dn \right) \quad (2)$$

where  $N$  is the total number of time intervals (bins) in the data set. We see that for  $\chi \rightarrow \infty$ , the number of false positive events  $K(\chi)$  approaches zero, as expected. For a finite threshold  $\chi$ ,  $K(\chi)$  is different from zero but can always be made sufficiently small by choosing the right value of  $\chi$ . We may for example define the threshold  $\hat{\chi}$  by the implicit equation

$$K(\hat{\chi}) = 1 \quad (3)$$

which corresponds to the detection of one false positive event in  $N$  data bins. Having reached this point, the problem of distinguishing a transient event from the background is reduced to the task of finding a sufficiently good estimate for the probability distribution of the background alone. To

find such an estimate we propose using an iterative method. In a first iteration, the original data set is used to calculate an estimate for the mean,  $\mu_1$ , and the standard deviation,  $\sigma_1$ , for the true  $\mu$  and  $\sigma$  that characterize the noise. Since  $\mu_1, \sigma_1$  are calculated for the entire data set including peaks well above the noise, we expect that  $\mu_1, \sigma_1$  overestimate the true  $\mu, \sigma$ . Assuming a Poisson distribution we fail - in this first iteration - to accurately fit the noise peak of the histogram  $H(n)$ . However we may still use  $\mu_1$  to obtain a first estimate for the noise distribution

$$P_1(n) = \frac{e^{-\mu_1} \mu_1^n}{n!} \quad (4)$$

which may then be used to calculate and estimate  $K_1(\chi)$  for the true  $K(\chi)$

$$K_1(\chi) = N \times \left( 1 - \int_{-\infty}^{\chi} P_1(n) dn \right). \quad (5)$$

Fig. 2 shows a plot of  $K_1(\chi)$  together with

$$K_{ns}(\chi) = N \times \left( 1 - \int_{-\infty}^{\chi} H(n) dn \right) \quad (6)$$

which is the analog of Eq. (2) however using the histogram  $H(n) = P(n) + S(n)$  of the time trace of Fig. 1 (b) instead of  $P(n)$  alone. Now  $K_1(\chi)$  is used to calculate a first estimate  $\hat{\chi}_1$  for the true value of  $\hat{\chi}$  by invoking the analogue to Eq. (3) for  $K_1(\chi)$ . The respective solution of (3) is visualized in the zoom of  $K_1(\chi)$  in Fig. 2.

Once a first threshold  $\hat{\chi}_1$  is determined, the next step consists in counting fluorescence bursts with countrates above  $\hat{\chi}_1$ . This task is performed using a Labview (NI) routine based on an algorithm that fits a quadratic polynomial to a sequence of data points. The main inputs of the routine are the threshold  $\hat{\chi}_1$  and the number of consecutive bins  $M$  to be used in the fit.

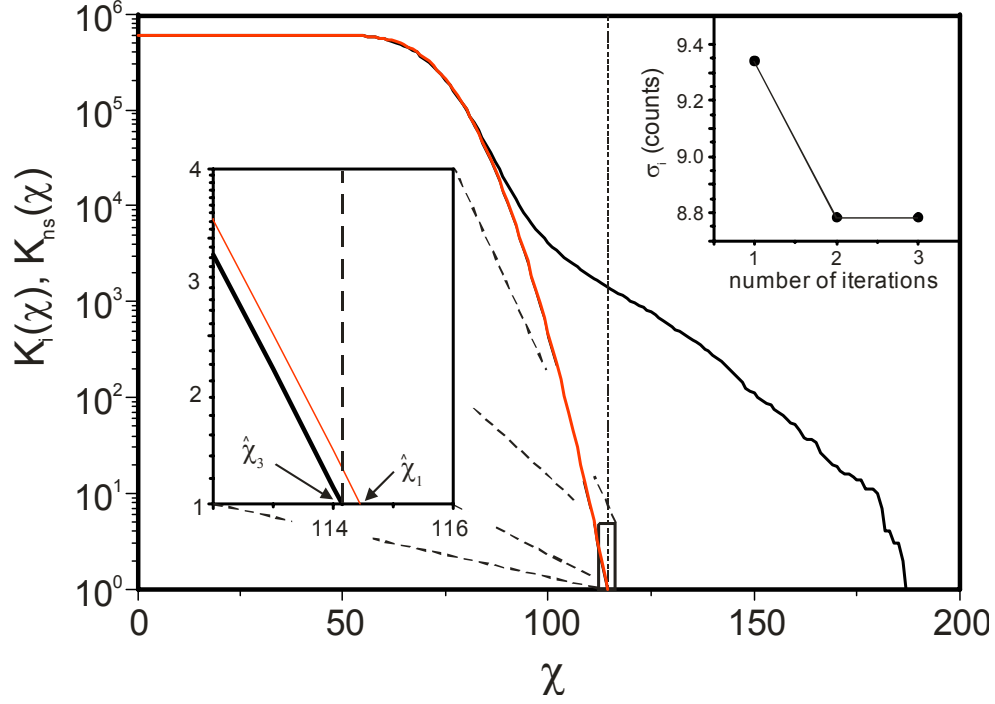
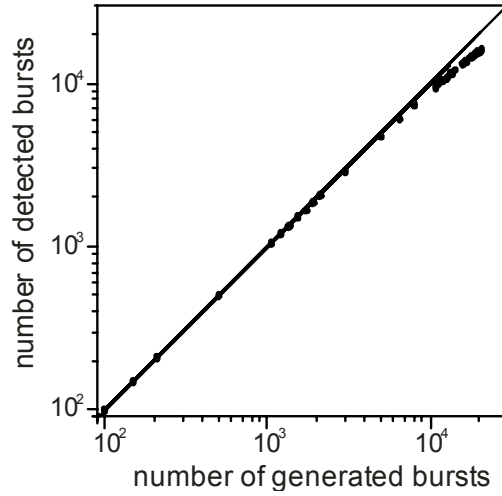


Fig. 2. Visualization of  $K_i(\chi)$ . Already the first estimate of  $K(\chi)$  obtained by calculating the mean of the time trace of Fig.1 provides a good estimate for the threshold. After three iterations all fluorescence bursts are eliminated. The mean of the remaining time trace perfectly characterizes the noise distribution.

Setting a small width  $M$  allows a finer resolution of the search for transient events but is prone to the detection of multiple peaks due to fluctuations on broader peaks. In contrary, a too large width prevents the detection of short bursts. To overcome the limitations of either situation, peak detection is performed as follows: the width is gradually decreased starting from a pre-defined maximum pixel number  $M$ . For each value of the width the number of detected bursts is stored. Each detected burst is then removed from the data by removing the respective bins. After  $M$  runs of the burst detection routine all bursts above  $\hat{\chi}_1$  have been counted and removed. The remaining data set now consists of the background noise plus a few peaks with amplitudes smaller than  $\hat{\chi}_1$ . In the second iteration step the truncated data set obtained in the first iteration is used to calculate new estimates,  $\mu_2$  and  $\sigma_2$  that better characterize the probability distribution of the noise. As a consequence,



more bursts are expected to be found in this second iteration step when applying the burst finding algorithm described before. After  $i$  iteration steps,  $\mu_i(\sigma_i)$  converges to a stable minimum  $\mu(\sigma)$ , which then provides a very good estimate for the parameter describing the true histogram of the background noise  $P(n)$  (Eq. 1). In practice it is found that the algorithm converges extremely fast. As can be seen in the inset of Fig. 2, the standard deviation of the truncated data set is stable already after 2 iterations. The zoom of  $K(\chi)$  in Fig. 2 shows that the final threshold  $\hat{\chi}_3$  is only marginally smaller than the first estimate. The resulting best estimate for the noise distribution using the parameter  $\mu_3$  is plotted in Fig. 1 together with histogram of the time trace. A remarkable agreement is found.



*Fig. 3. Monte-Carlo simulation of the burst detection process. The number of artificially generated bursts superimposed to a Poissonian noise is plotted against the number of bursts recovered by applying the burst detection algorithm. Bursts are faithfully recovered for burst densities up to several thousand bursts per trace. For higher burst densities significant overlap between bursts starts to diminish the number of recovered bursts.*

### **3.3 Discussion**

Finally, we apply a Monte-Carlo simulation of the burst counting process to investigate the reliability of the proposed algorithm. To this end we generate artificial time traces consisting of Poissonian noise with superimposed bursts of a fixed amplitude that are randomly distributed in time. The number of superimposed bursts is varied to assess the performance of the algorithm at high densities of bursts. To each generated trace the burst detection algorithm as described above is applied and the number of detected bursts is plotted versus the actual number of bursts. The result is displayed in Fig. 3. Obviously, the number of recovered bursts well recovered up to a several thousand bursts per trace. For larger numbers of bursts less bursts are recovered due to the onset of significant probability of overlap between bursts which are then counted as single event. We would like to stress the fact that the deviation observed is not a limitation of the presented algorithm but is a problem inherent to the type of data that are analysed. Monte-Carlo simulations can be used to determine correction factors to recover the actual number of bursts in applications that require very high precision and linearity.

### **3.4 Conclusion**

We introduced an algorithm that is able to faithfully recover transient events in the presence of significant stationary noise. The method is based on the determination of an optimal detection threshold that avoids the detection of false positive events while recovering most of transient events. Using the proposed algorithm recovery of single-molecule fluorescence bursts in presence of a strong Poissonian background was demonstrated. The method presented here provides the basis for the

analysis of single-molecule fluorescence burst data discussed in the following chapters.

Having achieved the ability to detect molecules in a solution, the next step of quantification seems to be at reach. Without proper adjustment of the setting parameters, however, a correct quantification of target molecules is virtually impossible.

In the next section the influence of various operation parameters and their mutual influences on the performance of the sensor will be discussed.

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## **4. Optimal operation conditions for remote sensing of fluorescence-labelled oligonucleotides in liquids through an optical waveguide**

### ***4.1 Introduction***

In chapter 2 we have introduced a biosensor that allows for rapid remote single fluorescent-labelled oligonucleotide molecules detection in a liquid environment at room temperatures through an optical waveguide. Here we discuss the influence of various operation parameters and their mutual influences on the performance of the sensor. The optimal operation conditions of the setup are investigated by varying the relevant parameters over a wide range. We find optimum values for the stirring velocity, the excitation intensity, the bin width and the experiment duration.

### ***4.2 Experimental***

All experimental hardware and settings were identical to the ones used in chapter 2, if not precised otherwise.

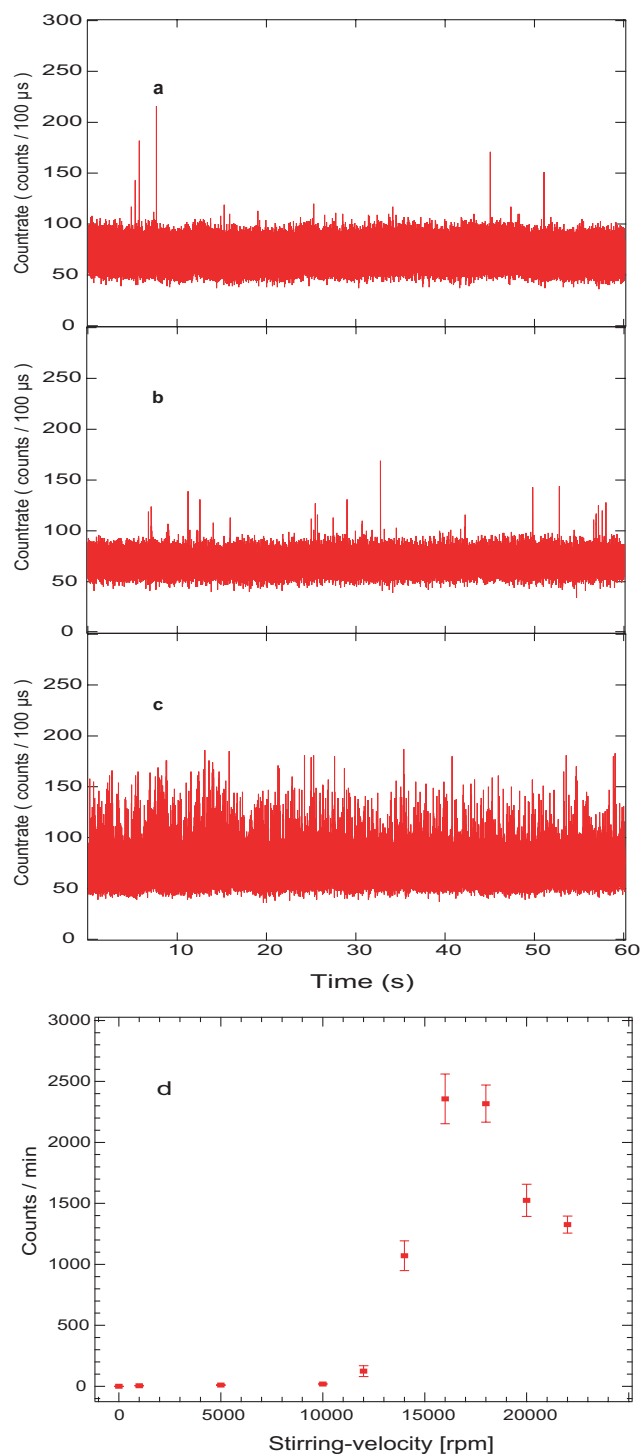
### ***4.3 Results and Discussion***

#### **4.3.1 Influence of the stirring rate**

The efficiency of the here proposed method of single-molecule detection and quantification method relies on the ability to acquire a maximum number of events per given duration of the experiment. The goal is to force the maximum number of target molecules to trespass the detection

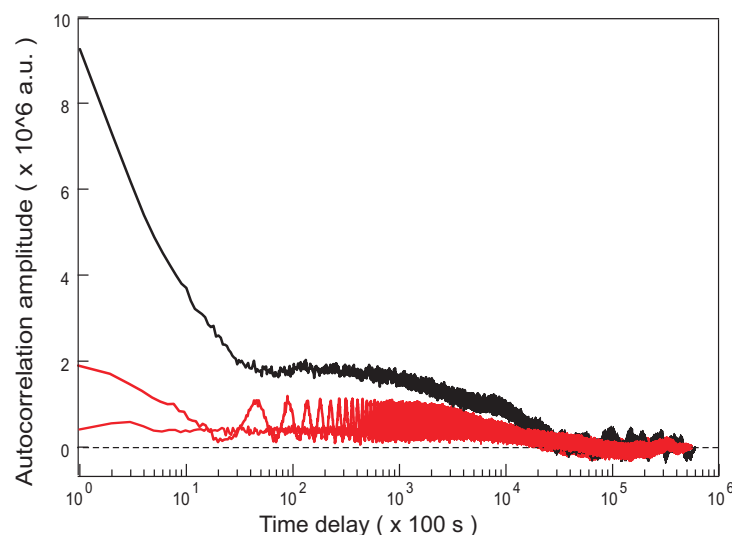
volume of the glass fiber sensor, but still enabling a sufficient number of fluorescence photons to be recorded during a bin width of about 100  $\mu$ sec. We apply stirring of a sample solution containing the target molecules (FRET (Cy5.5/Cy7) oligonucleotide ssDNA) to impose a constant flow of liquid across the detection volume. Fig. 1. shows different traces recorded at different stirring rates (low at 5000 rpm (a), medium at 12000 rpm (b), high at 16000 rpm (c)), all using the same excitation power (2 mW), bin width (100  $\mu$ sec) and same concentration of fluorescent target molecules 10 fM.

Two observations can be made comparing the different traces. First of all, the more obvious difference between these traces is the amount of single fluorescent events registered. The higher the stirring rate, the more events occur. This suggests that the higher the stirring rate, the higher the probability for the single target molecules to trespass the detection volume and to produce a fluorescence burst [see chapter 2.3 and 3.2]. The second remarkable observation concerns the amplitude of the different bursts. At the optimal stirring rate of 16000 rpm (see Fig. 1d) the amplitude of the majority of the single bursts becomes uniform and almost results in the same amplitude. This amplitude of approximately 50 counts above the background signal sufficiently well correlates with the saturation count rate for single molecules at a bin width of 100  $\mu$ sec [see chapter 2 and below, influence of the bin width], presuming the donor fluorophore (Cy5.5) of the FRET pair was saturated at an excitation power of 2 mW and a small fraction of the fluorescence of the acceptor fluorophore (Cy7) is directed into the fiber core [see chapter 2.3]. At a lower stirring rate (b) higher peaks and lower peaks than of a 50 counts amplitude can be observed.



*Fig. 1: Effect of different stirring rates on the number of fluorescence bursts. (a) was recorded at a stirring rate of 5000 rpm, (b) at 12000 rpm and (c) at 16000 rpm. (d): Number of fluorescence bursts per minute as a function of the stirring rate. Bin width 100  $\mu$ sec, excitation power 2 mW, concentration of target molecules 10 fM.*

Several target molecules which trespass the detection volume of the sensor simultaneously, provoke a collective signal. Fluorescent bursts of this origin generate much higher amplitude than 50 counts at a bin width of 100  $\mu$ sec. Hence, presuming the bin width for both traces being identical, a peak occurring in a time frame recorded at a high stirring rate should appear narrower than one recorded at a lower stirring rate, because of the decreased time available for the molecule to couple its fluorescence back into the fiber. In order to determine the typical duration of a fluorescence burst two time traces at different stirring rates each (12000 rpm and 15000 rpm, respectively) but the same bin width (100  $\mu$ sec) and concentration of target molecules 10 fM were subjected to an autocorrelation analysis (Fig. 2).



*Fig. 2. Autocorrelation of the fluorescence time trace as a function of time delay of Fig. 1 (b), 1 (c) and of a time trace which was recorded in plane buffer without target molecules. All autocorrelations with an excitation power at 2 mW and a concentration of 10 fM of target molecules, except for the negative control (2 mW, 16000 rpm, plane buffer solution).*



As a negative control an autocorrelation of a time trace recorded with a plain buffer solution, which contained no target molecules, was calculated as well. The autocorrelation shows a mean duration of single bursts of approximately 500  $\mu\text{sec}$  compatible with an optimum bin width of 100 $\mu\text{s}$ . The choice of the correct bin width will be discussed in the following section. It has to be mentioned that the detection algorithm does not differentiate between a high or low, expanded or narrow peak. They all will be counted as one burst regardless its amplitude or expansion [see chapter 2 and 3]. Collective signals will therefore decrease the total amount of fluorescent bursts recorded during a certain time frame.

In order to properly assess the correct stirring rate for a bin width of 100  $\mu\text{s}$  a series of experiments at a constant excitation power of 2 mW were performed. The glass fiber was dipped in a buffer solution containing fluorescent target molecules at a concentration of 20 fM. The acquisition time for each point was one minute. Five separated measurements were conducted at each stirring rate.

Fig. 1 (d) shows the result of the experiment. The optimal stirring rate seems to lie between 16000 rpm and 18000 rpm for the above-mentioned settings. It should be emphasized that poor or no detection of target molecules resulted from using stirring rates below 10000 rpm or no stirring at all, respectively. The intrinsic diffusion is too slow to exchange the whole detection volume in a reasonable amount of time [see chapter 2]. In addition it should be considered that a molecule traveling at such a low speed would probably be bleached instantaneously by the divergent excitation field exiting the fiber tip before it could couple its fluorescence signal into the glass fiber core [see chapter 2.2.7] [1-5]. Using a too high stirring rate (above 20000 rpm) resulted in a decrease of single fluorescence bursts per minute as

expected because of a too fast transition of the molecules through the excitation volume.

### **4.3.2 Influence of the bin width**

Data are recorded by counting the number of fluorescence counts that fall in subsequent bins of a certain finite length. Variations of this bin width have a strong influence on the quality and appearance of the data. For too short bin widths, the number of photons per bin decreases which in turn increases the relative importance of shot noise. For too long bins, the time resolution is no longer sufficient to resolve closely spaced peaks. An important aspect of the detection of fluorescence through an optical waveguide is the red-shifted background generated in the waveguide itself [see chapter 2.2.7] [6].

The longer the binning time, the higher the mean value of the background and the higher its absolute shot noise amplitude. Since the number of counts that may be extracted per time interval from single molecules is limited, any alteration of the background amplitude will have an impact on the signal-to-noise ratio. To obtain an estimate for the signal-to-noise ratio we assume the saturation count rate of a single molecule to be approximately  $500 \times 10^6$  photons per second [6]. Further assuming a detection efficiency of about 0.1% [see chapter 2.3.1] we determine the maximum number of photons recorded in one bin for a single fluorophore to equal 500 counts, 50 counts or 5 counts for binning time rates of 1 msec, 100  $\mu$ sec or 10  $\mu$ sec, respectively. Accordingly the signal to noise ratios for the corresponding bin widths can be elicited, considering the amplitude of the background noise of the corresponding bin. The results are documented in Table 1.

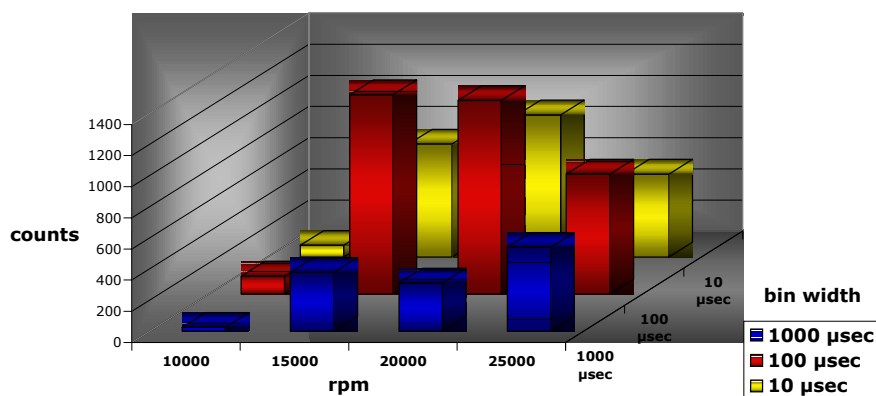
Bin width	Mean background counts per bin	Background noise amplitude	Calculated maximum counts per bin	Signal-to-noise ratio	Detected bursts per min
1000 $\mu$ sec	3450	59	500	8.5	378
100 $\mu$ sec	320	18	50	2.8	1281
10 $\mu$ sec	35	6	5	0.8	726

*Table 1: Comparison of bin widths for a fixed stirring rate of 17000 rpm, excitation intensity of 2 mW, and concentration of 5 fM fluorescent-labelled target oligonucleotide molecules.*

The signal to noise ratio for a binning of 1 msec would be considered optimal and thus would be expected to result in a maximum of single fluorescent bursts to be detected per time interval. However, experiments conducted with identical settings but variable bin width could not confirm this expectation. At an excitation intensity of 2 mW, a constant stirring rate of 17000 rpm and a concentration of 5 fM fluorescent-labelled target molecules, the highest number of fluorescence bursts was observed for 100  $\mu$ sec binning. Choosing a too short bin width decreases the signal to noise ratio and therefore result in a loss of the absolute number of counts during a given time frame of one minute because the bursts are prone to fall below the threshold. A too long bin width results in a lower number of detected fluorescence bursts since multiple peaks contribute to only a single burst. This regime should be avoided since the dynamic range of the measurement is being diminished.

The experiments discussed so far were performed at a fixed stirring rate of 16000 rpm and 17000 rpm, respectively. It is expected that the stirring influences the duration of single molecule fluorescence bursts since it influences the velocity at which target molecules pass the detection volume above the glass fiber core. To exclude any influences of the

stirring rate on the optimum bin width we have determined the number of fluorescence bursts in a solution containing fluorescent-labeled target molecules at a concentration of 5 fM for different stirring rates. In Fig. 3 the number of detected fluorescence bursts during one minute is plotted as a function of the stirring rate and the bin width. It is clearly visible that among all of the three bin widths investigated, 1 msec nearly consistently shows the lowest number of detected bursts. Surprisingly, for both 100  $\mu$ sec and 10  $\mu$ sec bin width a maximum count rate for target molecules during one minute is observed at a stirring rate between 15000 and 20000 rpm. Both bin widths show a decrease of count rates for target molecules at stirring rates towards 25000 rpm. It appears that at 25000 rpm or above a majority of molecules pass the detection volume too fast to yield a sufficient signal that could be properly discriminated from the background noise. However for both bin widths a maximum count rate for stirring rates between 15000 and 20000 rpm is observed.

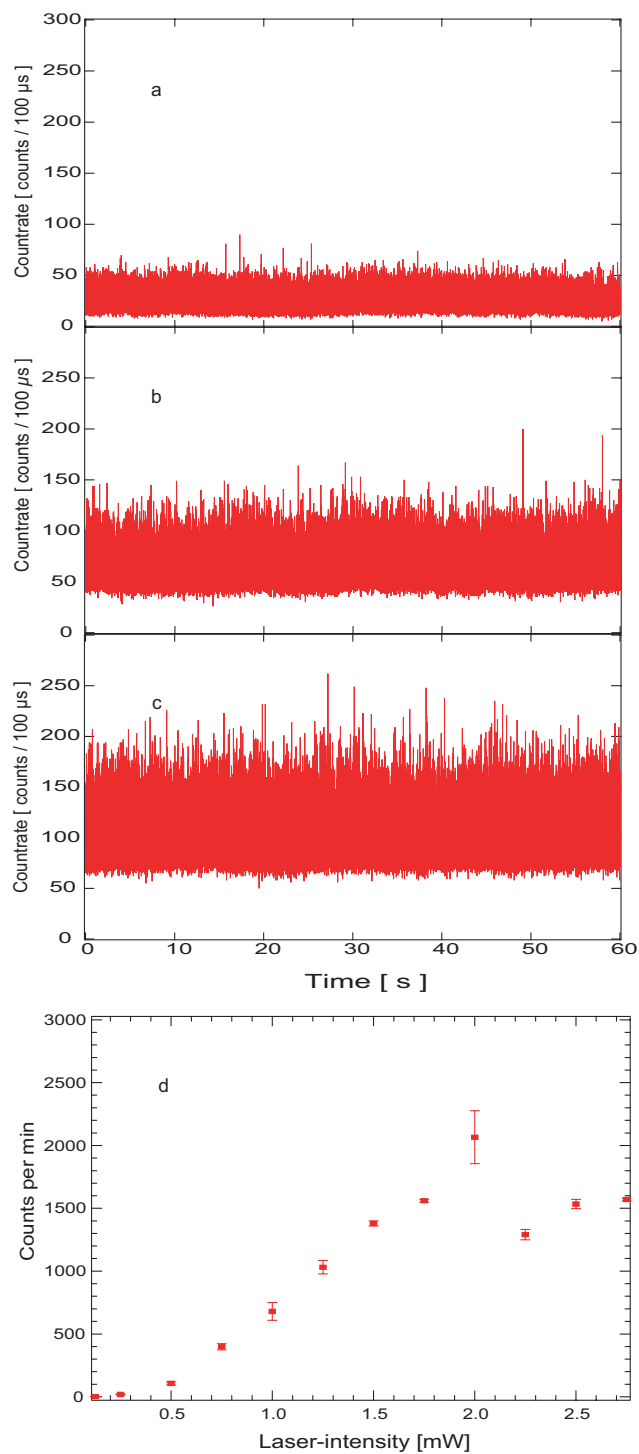


*Fig. 3: Number of bursts per minute as a function of stirring rate and bin width. Excitation power at 2 mW, concentration of 5 fM target molecules.*

### 4.3.3 Influence of the Excitation power

Since the emission of photons from individual molecules is subject to a saturation behavior [6] it is expected that the number of detected fluorescence bursts for a given duration of the experiment will go through a maximum. For low power the burst amplitude will increase linearly with the excitation power. The number of detected bursts above the threshold will slowly increase as well. Once all of the fluorescence bursts are saturated the number of detected peaks can no longer increase. As a matter of fact for further increased excitation power, the background will grow much faster than the fluorescence burst amplitude. Therefore the number of detected peaks will start to deteriorate.

Fig. 4 shows traces (a-c) obtained at various excitation intensities at a bin width of 100  $\mu$ sec, a stirring rate of 17000 rpm and a 10 fM concentration of fluorescent target molecules. The higher the excitation rates in these traces, the higher the mean count rate and the amplitude of the background noise. But remarkably the number of fluorescent bursts increases as well because more and more bursts are detected above the threshold. Fig. 4 (d) shows traces obtained at various excitation intensities at a bin width of 100  $\mu$ sec, a stirring rate of 17000 rpm and a concentration of 10 fM fluorescent-labelled target molecules for any measurement point. The measuring time for every point was one minute. Five separated measurements were conducted at every excitation rate to obtain a mean value. Fig. 4 (d) shows that a saturation exists for the needed excitation power beginning at a value of approximately 1.8 mW.



*Fig. 4 (a-c):Number of fluorescent bursts per minute as a function of time for 500  $\mu$ W (a), 1.2 mW (b) and 2 mW (c), respectively. Bin width at 100  $\mu$ sec, stirring rate at 17000 rpm, concentration of target molecules 10 fM. (d): Number of fluorescent burst per minute as a function of excitation power. Bin width at 100  $\mu$ sec, stirring rate at 17000 rpm, concentration of target molecules*

10 fM. Note the increasing amplitudes of background and fluorescence bursts with increasing excitation power.

In order to obtain the maximum number of single fluorescent bursts during a fixed time frame it is mandatory to excite the fluorophore FRET pair with a proper excitation power to achieve saturation. For typical fluorophores an intensity of 1 kW/cm<sup>2</sup> is generally considered to be a good estimate for the saturation intensity for which half of the saturation count rate of a fluorophore is achieved (Fig. 5) [6]. However, to fully saturate a molecule it is necessary to excite at intensities that are up to 10 times larger than the saturation intensity. The diameter of a light guiding glass fiber core at a wavelength of 633 nm measures approximately 4 µm in diameter according to the manufacturer (see chapter 2.2.7). For this diameter, 10 kW/cm<sup>2</sup> translate into a power of approximately 1.264 mW. Remarkably, the value needed for the maximum amount of fluorescent bursts per minute as seen in Fig. 4 (d) is in the range of 1.8 mW, compatible with the observed saturation in Fig. 5.

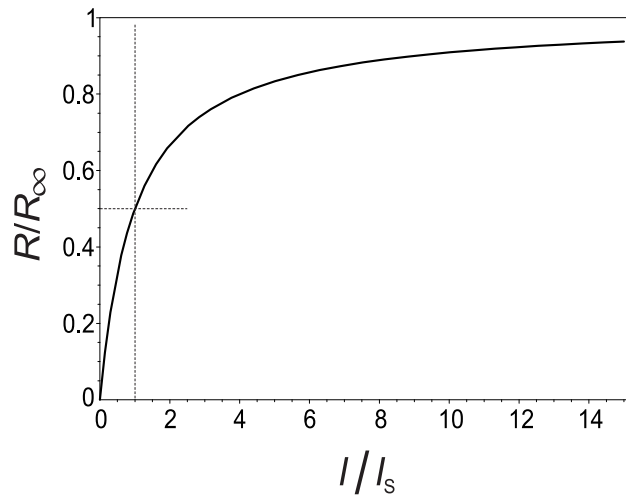
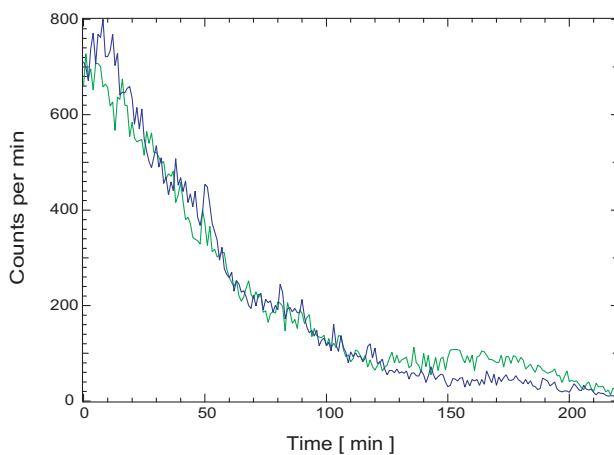


Fig. 5: Saturation of the emission rate of a single molecule as a function of the excitation intensity.

#### 4.3.4 Influence of the measuring time

Another crucial parameter is the role of photo-bleaching in all experiments. Several bleaching experiments were conducted in which different solutions containing the same concentration of fluorescent target molecules at 10 fM were continuously excited at a rate of 2 mW over a period of nearly four hours. The stirring rate for these experiments was at 17000 rpm and a binning time of 100  $\mu$ sec was used. As shown in Fig. 6, the exponential decrease of the fluorescence yield starts almost immediately due to bleaching, yet remains approximately stable during the first five minutes. Therefore the most beneficial moment to record a time trace is immediately after the stirring has been initiated. The maximal time span to acquire single fluorescent signals is 5 minutes.



*Fig. 6. Photobleaching curves of two solutions containing the same concentration of fluorescent labelled target molecules at 10 fM. Stirring rate for these experiments was 17000 rpm and binning time of 100  $\mu$ sec was used. The photobleaching starts almost immediately yet remains approximately stable during the first five minutes.*



## **4.4 Conclusions**

Exploring the setting characteristics and their interactions of a new detection system is crucial if a reliable detection and quantification method is to be established. Our investigations demonstrate the importance of the correct choice of settings for this novel single-molecule detection method.

The integration time of 100  $\mu$ sec to enable the detection of single fluorescence molecule events in liquids that was found. The mean duration of a single burst is approximately 500  $\mu$ sec. The stirring rate also plays a crucial role for this detection method. We were able to demonstrate that the dependence on the stirring velocity shows a maximum at a range between 16000 rpm and 18000 rpm and that stirring at low rates yields almost no signal at all. Additionally we could exclude any reciprocal interactions between the bin width and the stirring rate. Our experiments further demonstrated that the excitation power at the end of the detecting glass fiber needed to be in the order of ten-fold higher than could be anticipated by rough calculations concerning the saturation of single fluorophores and lies in the range of 2 mW. We also could show that the most favorable time span to acquire single fluorescent bursts from a sample solution is during the very first 5 minutes if continuously illuminated.

## 4.5 References

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## **5. Quantification of wavelength-shifting fluorescence-labelled oligonucleotides in liquids through an optical waveguide**

### ***5.1 Introduction***

After extensive investigation of the optical sensor's setting parameters and its potential for single molecule detection of fluorescent target molecules [see chapter 4], we now aim to demonstrate its capabilities for the rapid quantification of target molecules in the range of minutes.

Single-molecule detection of wavelength-shifting fluorescence labelled oligonucleotides in liquids through a single-mode fiber is applied using previously determined optimal detection conditions [see chapter 4.3]. We demonstrate a linear dependence of the number of detected fluorescence bursts on the concentration of the test solution over a wide dynamic range starting at 100fM down to 1aM concentrations. This qualifies the apparatus to be applied in quantitative sensing application, e.g. in medical diagnostics.

### ***5.2 Experimental***

All experimental hardware and settings were identical to the ones used in chapter 2, if not precised otherwise.

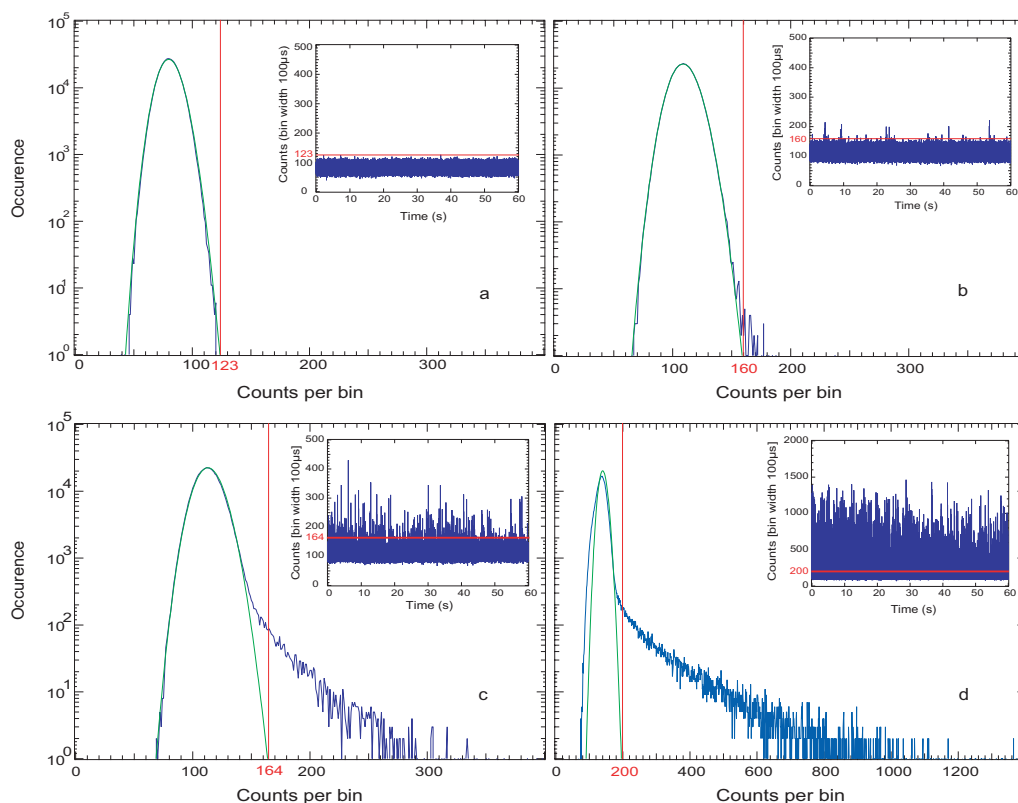
#### **5.2.1 Dilution procedure**

A dilution step was performed as follows. In a first step 1/10 (0.1 ml) of the total sample volume (1 ml) was pipetted out of the plexiglass fluid cell and kept in the pipette. The rest of the sample volume was then removed completely from the fluid cell. In a second step the fluid cell

was thoroughly rinsed with isopropanol (Sigma) and distilled water for 15 minutes. In a third step the parts of the optical setup that had been in contact with the sample were intensively rinsed with distilled water. In a fourth step the sample that was kept in the pipette (0.1 ml) was reinserted into the fluid cell and 9/10 (0.9 ml) of fresh buffer solution was added.

### ***5.3 Results and discussion***

In order to adequately being able to distinguish between fluorescent signals due to labelled target molecules and the strong background noise generated by the experimental setup itself [see chapter 2.3 and 4.2], several blank measurements were conducted. Such a measurement is shown in Fig. 1 (a). The luminescence trace of one minute (inset of Fig. 1 (a)) acquired in pure buffer in the absence of fluorescent-labelled oligonucleotides shows no single molecule events. The threshold is chosen such that for a given length of the time trace the probability for finding a peak above the threshold is negligible [see chapters 2.3 and 3.3]. The histogram of Fig. 1 (a) represents the Gaussian or Poissonian distribution of the background noise [see chapter 3.1].



*Fig. 1: Histograms of the occurrence of fluorescence bursts acquired during one minute of measurements. The insets present the time traces recorded for one minute. (a) plane buffer solution containing no fluorescent-labelled target molecules. Concentrations of fluorescent-labelled oligonucleotides: (b) 100 aM, (c) 10 fM, (d) 1 pM. Settings: Excitation power 2 mW, bin width 100  $\mu$ sec, stirring rate 17000 rpm.*

At a low concentration 100 aM of fluorescent-labelled target molecules single bursts can be perceived in the time trace (Fig. 1 (b, inset)). The bursts clearly pierce through the threshold and can thus be detected and quantified. The histogram of Fig. 1 (b) visualizes the distribution of the amplitude of the fluorescent signals. The majority of them have a count rate of about 50 counts per bin width (100  $\mu$ sec), which correlates approximately with the number of fluorescence photons emitted by one fluorophore for near saturation [see chapter 4], presuming a detection efficiency of 0.1 % [see chapter 2.3]. The background signal is also slightly altered. Whereas the mean count rate per bin width (100  $\mu$ sec)

was at 80 counts for the plane buffer solution containing no target molecules (Fig. 1 (a)), the mean count rate for the background noise at a concentration of 100 aM is at 110 counts (Fig. 1 (b)). Additionally the amplitude of the background noise has also increased. Approximately 80 counts for the buffer solution and 95 counts for the concentration of 100 aM. This is due to the fact that the fluorescence of target molecules is contributing to the background noise although they are not trespassing the detection volume of the sensor tip themselves. This fluorescence is also registered but does not contribute to fluorescent bursts [see chapters 2.3 and 4.3].

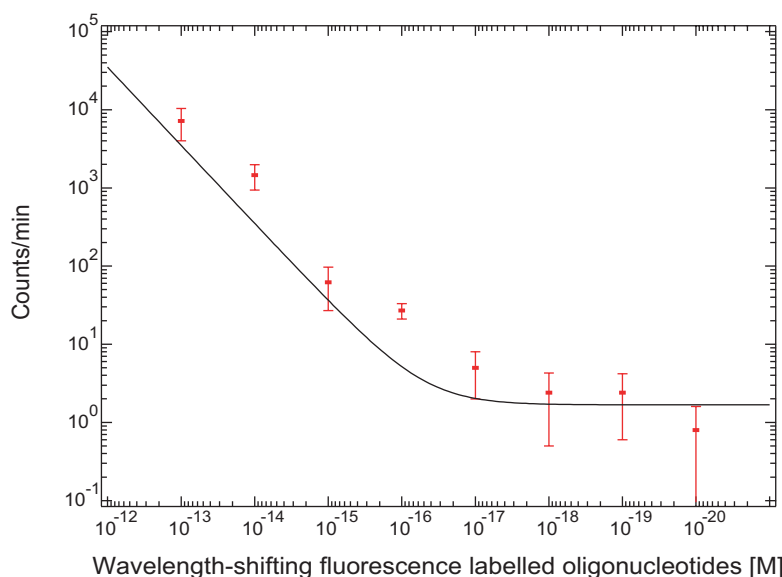
At a medium concentration of 10 fM target molecules (Fig. 1 (c), inset) the trace changes significantly, compared to the previous one. The most obvious difference is the amount of bursts registered during this one minute, which already reveals the expected dependence on concentration. In addition, however, the fluorescent bursts also increase in amplitude. This is due to the fact that at higher concentrations the probability of several target molecules to contribute to a collective signal is higher than for lower concentrations [see chapters 2.3 and 4.3]. The fluorescence generated from several target molecules, which trespass the detection volume of the sensor simultaneously, is added up and thus results in a much higher count rate than for single molecules [see chapters 2.3 and 4.3]. The background signal changes compared to the background for the plane buffer measurement for the above-mentioned reasons. Interestingly the fluorescence of this increased concentration (Fig. 1 (c)) does not contribute significantly to increase the mean count rate or amplitude of the background trace compared to the background at a concentration of 100 aM (Fig. 1 (b)). Note only the slight increase of the threshold for the concentration of 100 aM (at 160 counts) compared to the threshold of 10 fM (at 164 counts).

For a high concentration of 1 pM of target molecules however, the trace and the histogram change dramatically (Fig. 1 (d)) for the above-mentioned reasons.

We observe an extreme increase of the amount, as well as the amplitude of the fluorescent bursts. Most of the fluorescent bursts consist of collective signals. The mean count rate is now at 140 counts per 100  $\mu$ sec and the background amplitude even exceeds 120 counts per bin. Due to the Poissonian fit of the algorithm the threshold for 1 pM is set at 200 counts.

Comparing all of the histograms with their correlation time traces (Fig. 1 a-d), high fluorescence bursts appear much more visible in the time traces than in the histograms. This is an artifact of the display. Nevertheless does Fig. 1 clearly visualize that through the counting of fluorescent bursts originating from labeled target molecules over a time frame of one minute a quantification of target molecules is possible.

In order to properly assess the range of our detection and quantification method a dilution series was conducted. Fig. 2 shows the count rates per bin width (100  $\mu$ sec) obtained during one minute of recording as a function of the concentration of target molecules. The dilution series was started at a concentration of 100 fM and was conducted for 7 dilution steps, each step diluting the previous concentration by a factor of 10, until a concentration of 10 zM was reached. Already at a concentration of 1 zM the number of fluorescent molecules in volume of  $1\mu\text{m}^3$  is less than one. For the ultra-low concentrations discussed here, stirring of the solution is important to transport molecules into the detection volume.



*Figure 2: Counts per minute as a function of concentration of fluorescent-labelled target molecules. The measuring time for every point was one minute. Five separated measurements were conducted at every concentration to obtain a mean value. Settings: Excitation power 2 mW, bin width 100  $\mu$ sec, stirring rate 17000 rpm. The fit-function applied was  $y = ax+b$ .*

Every point in Fig. 2 represents a number of fluorescence bursts per acquisition interval for a given concentration averaged over 5 acquisition intervals. The acquisition interval for every point was one minute. Fig. 2 visualizes the linear concentration dependence of the fluorescent burst count rate gained during one minute of recording. This linear behavior starts at 1 pM and levels off at 1 aM. There is still a detection-sensitivity claimable in the zeptomolar range, a valid quantification however for the sub-attomolar range in 1 minute could not be achieved yet. For longer integration times this should however be possible. For high concentrations in the range of 1 picomolar, as was shown in chapter 3.3, it is often the case, that several target molecules produce a collective signal, which is counted as a single burst. This decreases the number of potentially measurable counts per time frame and explains the tendency towards higher concentrations to underestimate the true concentration. For lower concentrations however, this detection and quantification



method proofs to be highly proficient and accurate, since the probability for collective signals is negligible. We emphasize that only one minute is needed to perform a quantification of target molecules. The measurements take place in a liquid environment at room temperature and require no specialized lab equipment. Neither any amplification procedures, such as PCR, nor any pretreatment steps of the sample solution, such as a previous adsorption to a sensor part, are necessary. Additionally the sensor is able to operate with free-floating targets.

## **5.4 Conclusions**

The performed dilution series visualizes the linear dependency of the number of fluorescent bursts recorded during a time frame as a function of their concentration. The efficiency of this quantification system ranges from a concentration of 100 fM to 1 aM. It further could be demonstrated that our sensor shows accurate sensitivity for detection of single molecules in the range of zeptomolar concentrations.

## **5.6 Outlook**

In the next chapter the now established system of optical hardware and algorithm will be combined with molecular beacons. This will insert a molecular switch function into the system. The molecular beacons will operate completely free-floating and be remotely monitored through the glass fiber by the sensor.

## **6. Detection and quantification of molecular beacons in liquids via an optical waveguide**

### ***6.1 Introduction***

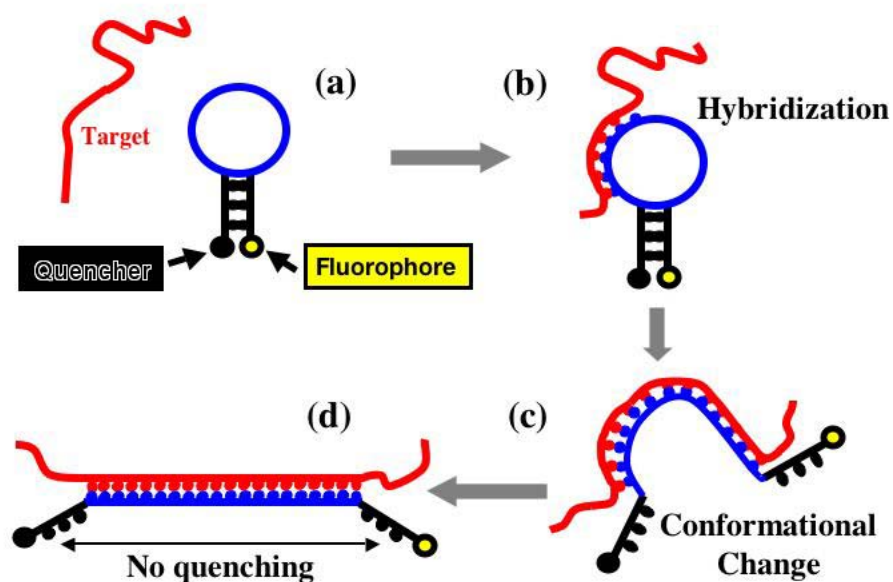
Based on the combination of our ultrasensitive fiber-optical detection system [see chapter 2-5] and smart fluorescent probes, i.e. wavelength shifting molecular beacons [1], we propose a novel approach for the detection and quantification of single unlabeled target RNA sequences in liquids. The detection system can be adapted to other relevant biological materials, like proteins, using adequate smart probes based on fluorescence. The combination of single-molecule sensitivity and detection through optical waveguides opens the road for rapid, reliable, ultra-sensitive, and cheap medical diagnostics by direct detection of relevant molecular markers.

In the following chapter we will demonstrate the application of molecular beacons, for the specific detection and quantification of characteristic Her2-mRNA [2-7] sequences in a test solution. In bulk experiments, the performance of the molecular beacons is checked. It is found that single base pair mismatches between beacon and target sequence can be detected through the analysis of melting curves. Single-molecule experiments with molecular beacons in the absence of targets show that only a negligible fraction of the beacons is open at room temperature and are detected as fluorescence peaks. Upon addition of perfect targets the number of detected fluorescence peaks increases dramatically. A linear dependence of the number of fluorescence peaks as a function of the concentration of molecular beacon-target sequence duplexes is observed. More remarkably, for a fixed concentration of molecular beacons, we observe a linear increase of the number of fluorescence peaks as a function of the target sequence concentration,

demonstrating the potential of the technique for the quantitative determination of low-concentrations of analyte molecules.

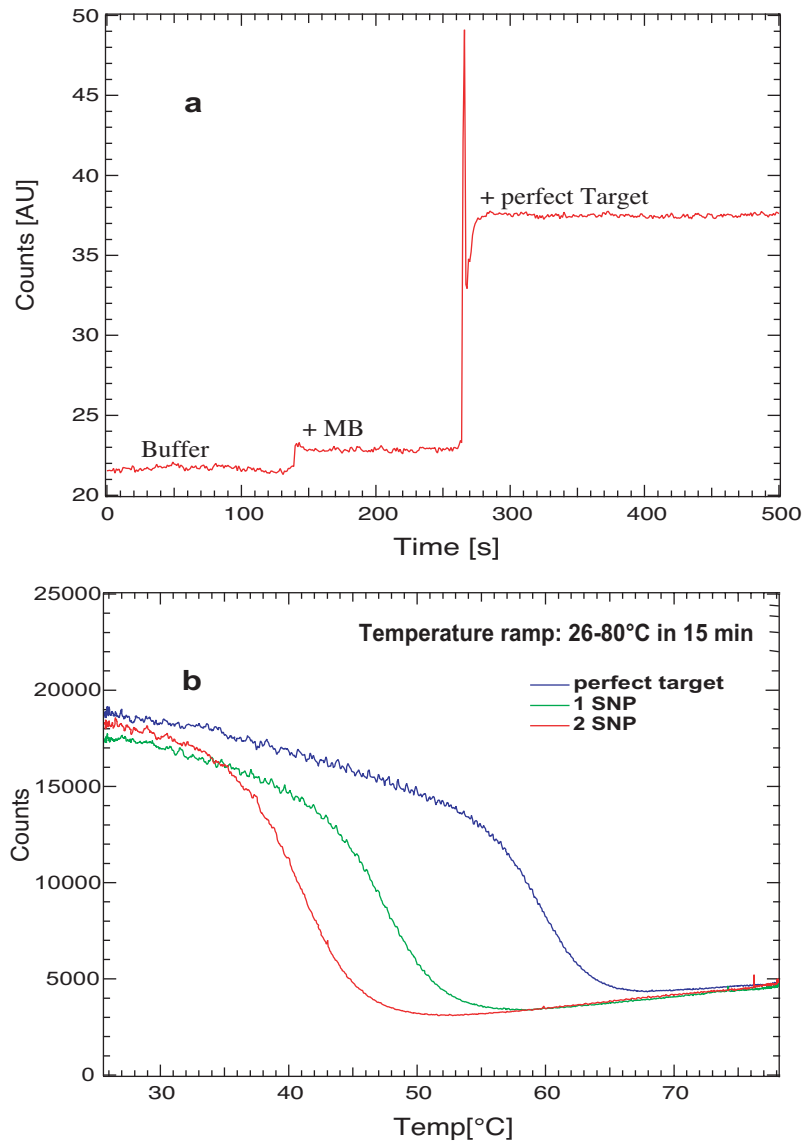
## **6.2 Molecular Beacons**

Molecular beacons (MB) are single-stranded DNA hybridization probes that form a stem-and-loop structure [8,9]. The loop contains a probe sequence (typical length between 20 to 40 bases) that is complementary to a target sequence, and the stem is formed by the hybridization of complementary arm sequences that are located on either side of the probe sequence. A fluorophore is covalently linked to the end of one arm and a quencher is covalently linked to the end of the other arm. Molecular beacons do not fluoresce when they are free in solution. However, when they hybridize to a nucleic acid strand containing a target sequence they undergo a conformational change that enables them to fluoresce brightly (Fig. 1).



*Fig. 1: Working principle of MB (a) In the absence of a complementary target the MB remains closed and no fluorescence is emitted. (b-d) When the MB hybridizes to a target molecule, it undergoes a spontaneous conformational change that forces the stem sequences apart and causes the fluorophore and quencher to move away from each other. Since the fluorophore is no longer in close proximity to the quencher, it fluoresces when excited by light.*

In the absence of targets, the probe is dark, because the stem places the fluorophore so close to the nonfluorescent quencher that they transiently share electrons, eliminating the ability of the fluorophore to fluoresce [8,9]. When the probe encounters a target molecule, it forms a probe-target hybrid that is longer and more stable than the stem hybrid. The rigidity and length of the probe-target hybrid precludes the simultaneous existence of the stem hybrid. Consequently, the molecular beacon undergoes a spontaneous conformational reorganization that forces the stem hybrid to dissociate and the fluorophore and the quencher to move away from each other, thereby restoring fluorescence (Fig. 1(d)).



*Fig. 2. Fluorescence measurement using a Photomultiplier (PMT) and wavelength shifting molecular beacons. (a) Addition of an oligonucleotide complementary to the MB sequence resulted in a massive increase in fluorescence as expected. The peak signal is an experimental artifact resulting from the pipet tip during injection of the target mRNA. (b) Melting curves of MBs hybridized to different target mRNAs with no (blue), one (green) and two (red) single base mismatches (single nucleotide polymorphisms, SNPs). The temperature ramp ran from 26°C to 80°C in 15 min. This experiment demonstrates the high specificity of the MB.*

Using MBs even allows for the discrimination of single nucleotide mismatches of target sequences if applied in a temperature controlled environment [10]. Molecular beacons are uniquely suited for the detection of single-nucleotide variations because they recognize their targets with significantly higher specificity than conventional oligonucleotide probes [9,10,11]. Their high specificity is a consequence of their stem-and-loop structure. When a molecular beacon binds to its target sequence, the formation of the probe-target hybrid occurs at the expense of the stem hybrid. Molecular beacons can be designed so that over a temperature range of a few degrees C, only perfectly complementary probe-target hybrids are sufficiently stable to force open the stem hybrid (see Fig. 2 (b)). Mismatched probe-target hybrids will not form, except at substantially lower temperatures. Therefore, a relatively wide range of temperatures exist in which perfectly complementary probe-target hybrids elicit a fluorogenic response, while mismatched molecular beacons remain dark. Consequently, assays using molecular beacons robustly discriminate targets that differ from one another by as little as a single nucleotide substitution [10].

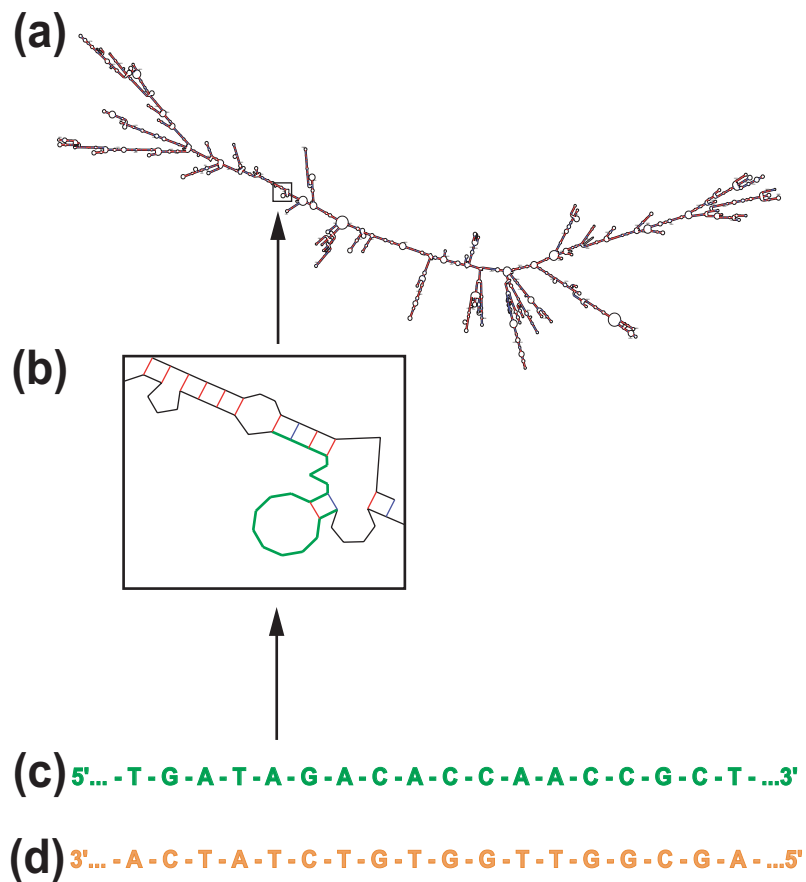
## **6.3 Experimental**

### **6.3.1 Design of HER-2 mRNA specific Wavelength-shifting MB**

Molecular beacons and targets were purchased from Genelink (Hawthorne, California, USA). We took a two-step approach to design suitable wavelength-shifting MB.

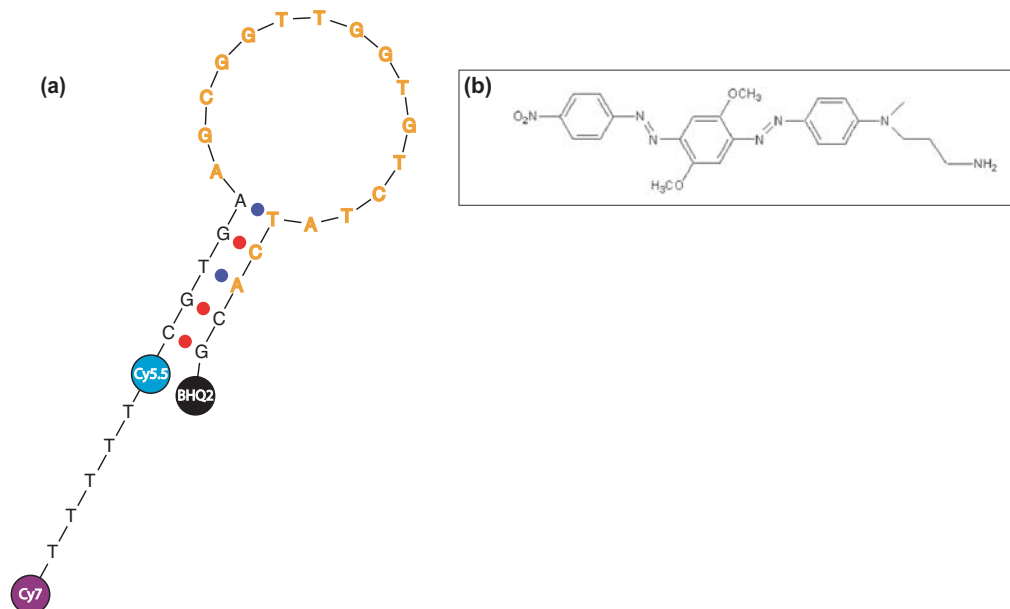
For a molecular beacon to be able to find its target sequence in a particular mRNA molecule, the target sequence should not lie within a tight secondary structure or be bound to proteins. Using the RNA secondary structure prediction program MFOLD [12], we identified regions in HER2 mRNA that were either single stranded or were paired

with distant sequences only in most of the thermodynamically favored foldings of HER2 mRNA. We then narrowed the choice of target regions with the help of a second computer program, OLIGOWALK [13], which identifies probe sequences that bind most stably to their complements and cause the least disruption in RNA secondary structure upon binding. The probe sequence with the best binding properties was then selected as the loop sequence for the molecular beacon (Fig. 3 (a-d)).



*Fig. 3. (a) Secondary structure of the linear HER2 mRNA (length: 4530 bases). (b) Region in the HER2 mRNA with favorable properties for MB hybridization (These should be preferentially single stranded, or at least paired with distant sequences only, in most of the thermodynamically favored conformations of HER2 mRNA). (c) Preferred mRNA sequence. (d) Probe sequence, that binds most stably to the preferred mRNA sequence.*

In order to bypass autofluorescence excited in the waveguide, wavelength shifting MBs were used. In these structures resonant energy transfer between a donor and acceptor dye is employed to create a huge Stokes shift of the acceptor emission with respect to the donor excitation. The FRET pair consisted of Cy5.5 and Cy7 [14-17]. BHQ2 was chosen as quencher for the Cy5.5 donor fluorophore [14-17] (Fig. 4 (a)). BHQ dyes function as efficient dark quenchers over the entire visible spectrum and into the near-IR, re-emitting their energy as heat rather than light. Probes made with BHQ dyes exhibit extremely low background fluorescence, enabling enhanced detection sensitivity.



**Figure 4** (a) A wavelength-shifting molecular beacon contains three labels: a quencher moiety at the end of its 3' arm sequence (BHQ2), a donor fluorophore at an internal location in its 5' arm that is opposite to the quencher in the hairpin conformation (Cy5.5), and an acceptor (emitter) fluorophore at the distal end of its 5' arm (Cy7). The probe sequence (orange color) is complementary to a target sequence of the Her2 mRNA. (b) Shows the structure formula of the dark quencher BHQ2 (Absorbance max.: 579nm, quenching range: 550-650 nm)

The donor fluorophore is selected to efficiently absorb energy from the available monochromatic light source. In the absence of targets, these



probes are dark, because the energy absorbed by the donor fluorophore is rapidly transferred to the quencher and transformed in heat. In the presence of targets, molecular beacons undergo a conformational reorganization caused by the rigidity of the probe–target duplex, which forcibly separates the 5' arm from the 3' arm. In the target-bound conformation, the energy absorbed by the donor fluorophore is transferred by Fluorescence Resonance Energy Transfer (FRET) to the acceptor fluorophore, which then emits the energy as fluorescent light with a higher wavelength as compared to the wavelength of the excitation light. One limitation of conventional MB was that the optimal emission wavelength was usually only a few nanometers longer than the optimal excitation wavelength. Consequently, a portion of the excitation light could reach the detector by scattering and reflection, thus limiting detection sensitivity. The large shifts of wavelength-shifting MB, allow more effective filtering of the excitation light, thereby enhancing the sensitivity of target detection.

It should be emphasized that the fluorescence signal measured by the photomultiplier (PMT) (J&M, Aachen, Germany), consists of the overall fluorescence yield of all the MB in the open state. In comparison to the fluorescence signal measured by the PMT shown in Figure 1(a), the fluorescence signal acquired with the APD of the optical setup consists of single molecule events. Hereby the photon yield of all open MBs passing by the glass fiber core during one bin correlates to the value of one event. The same experiment that was performed with the PMT (described in Fig. 1a) could be reproduced.

### 6.3.2 Design of synthetic targets

Synthetic targets for the wavelength-shifting MB were purchased at Genelink. The 18 nucleotide long sequence was complementary to the probe sequence of the wavelength-shifting MB. For the melting experiments, targets with one and two SNPs were synthesized.

*Sequences:*

Perfect target: TGATAGACACCAACCGCT

Target with 1 SNP: TGATAGACA**A**CAACCGCT

Target with 2 SNP: TGATAGACA**AA**AACCGCT

### 6.3.3 Hybridization Buffer

The buffer used in all experiments contained 10mM Tris-HCl, pH8.3, 50mM KCl, 1.5mM MgCl<sub>2</sub> and Ultrapure RNase-free water (Sigma). All experiments were conducted at 26°C +/- 0°C (regulated by a Labview feedback loop using a thermocoupler, F12 MVCH, Julabo Labortechnik GmbH, Seelbach Germany).

### 6.3.4 Optical setup

All experimental hardware and settings were identical to the ones described and determined in chapters 4 and 5, if not specified otherwise.

## 6.4 Results and Discussion

### 6.4.1 Detection of single wavelength-shifting molecular beacons

As previously shown (see chapters 2-5) single molecule fluorescence events resulting from fluorescence-labelled oligonucleotides may be discriminated from a strong normally distributed background. Also, quenching of the FRET signal of the wavelength-shifting molecular beacons is sufficiently strong to allow for a clear discrimination between hybridized and unhybridized MBs. The background signal of the buffer as shown in inset of Fig. 6a results only from autofluorescence occurring in the optical setup (mean value of the background signal is 700 counts at a bin width of 100 $\mu$ s). Neither the buffer nor the synthesized targets contribute to an increase of the background signal, as expected. Addition of wavelength-shifting molecular beacons to the buffer solution in the absence of complementary targets results in a slight increase of the overall background (<1%, mean value of the background signal: 706 counts at a bin width of 100 $\mu$ s), a phenomenon previously observed in experiments with solutions containing fluorophore concentrations in the pM range and above (see chapter 5.3). The increase of the background is due on the one hand to imperfect quenching capacity of BHQ2, (quenching efficiency for Cy5.5 is 95% [14]) and on the other hand due to the fact that the probability to find beacons in an open-state at the measurement temperature is not negligible.

The fluorescence  $F$  of the solution containing only the molecular beacons can be written as follows [10]:

$$F = \alpha \frac{[B_{closed}]}{B_0} + \beta \frac{[B_{open}]}{B_0} \quad (1)$$

Where  $[B_{closed}]$  and  $[B_{open}]$  are the concentrations of closed, respectively open beacons with  $B_0 = [B_{closed}] + [B_{open}]$  and  $\alpha$  and  $\beta$  are the characteristic fluorescence intensities of the molecular beacons in the

closed and open states, respectively. The equilibrium constant  $K$  for the opening of the stem loop depends on the temperature  $T$  and is given by  $K=[B_{\text{open}}]/[B_{\text{closed}}]$ . Using Eq. 1, we have

$$K(T) = \frac{F(T) - \alpha}{\beta - F(T)} \quad (2)$$

From the measurements shown in Fig. 5b, we can therefore extract the equilibrium constant  $K(T)$  giving the ratio of open to closed beacons. The Gibbs free energy change describing the closed to open state transition of the molecular beacons is given  $\Delta G = \Delta H - T\Delta S = -RT \ln K(T)$ . This approach supposes an all-or-none transition with temperature-independent enthalpy and entropy.

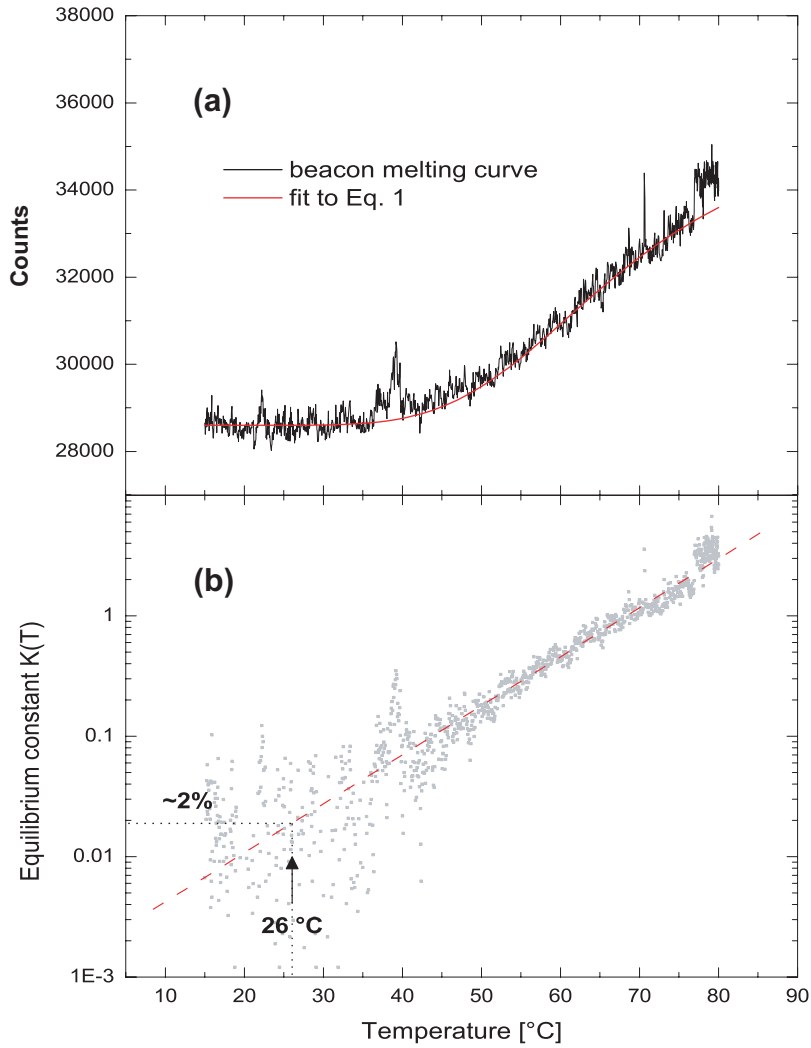
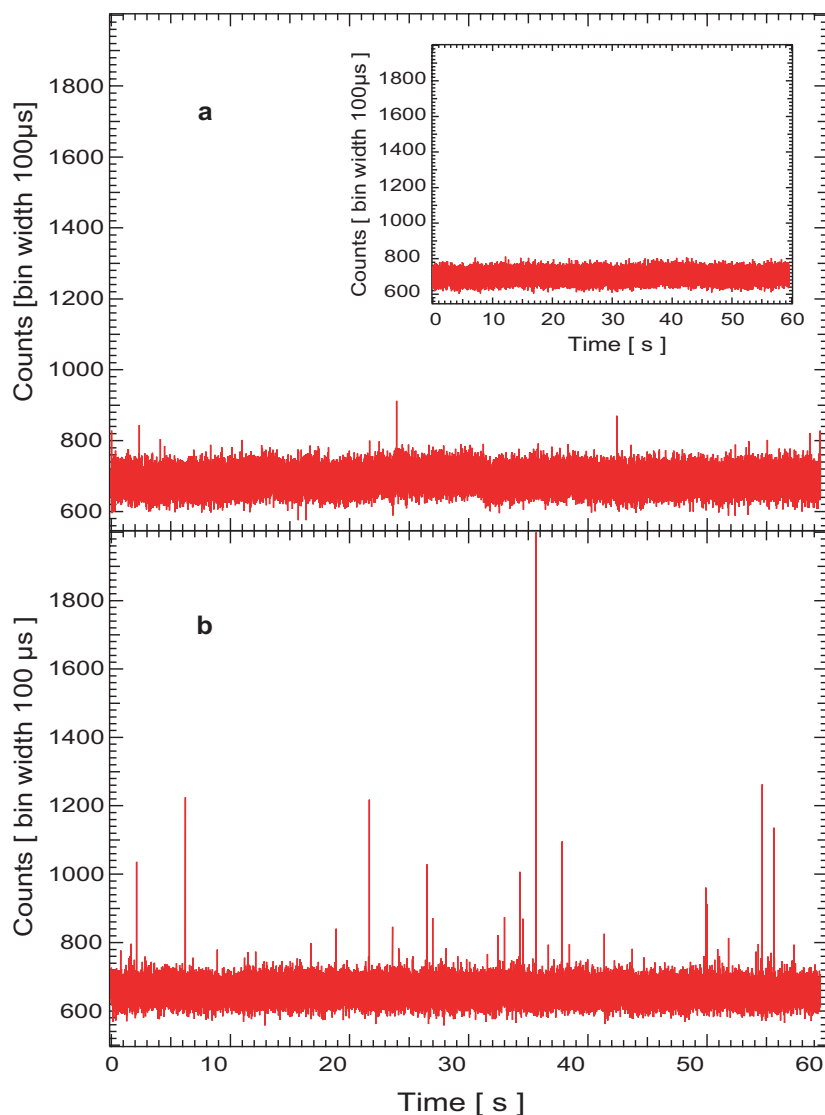


Fig. 5(a) shows a fit to the melting curve of wavelength-shifting molecular beacons. (b) Shows the equilibrium constant calculated from Eq. 2, using the parameters  $\alpha$  and  $\beta$  obtained from the fit. .

A rough estimate of the relevant thermodynamic parameters of the experiment [10] shows that we shall expect about 1-10% of opened beacons at 26°C due to thermal activation. This is in good agreement with the experimental observations. Indeed, the ratio  $r$  obtained by dividing the number of fluorescence peaks per minute for open beacons in the absence of complementary targets (Fig. 6a) by the number of fluorescence peaks for open beacons hybridized with perfect targets in excess (Fig. 6b) yields  $r = 0.09$ .



*Fig. 6: (a) Trace of wavelength-shifting molecular beacons in buffer in the absence of complementary targets. Inset in Figure 2(a): Background fluorescence of pure buffer. (b) Trace of hybridized wavelength-shifting molecular beacons. Settings as described in chapter 5.*

Neither the slight increase of the background nor the small number of bursts observed in absence of targets prohibit the detection and quantification of single-molecule events. Figure 6b shows a trace resulting from hybridization of perfect targets to wavelength-shifting molecular beacons after a hybridization time of ten minutes at a

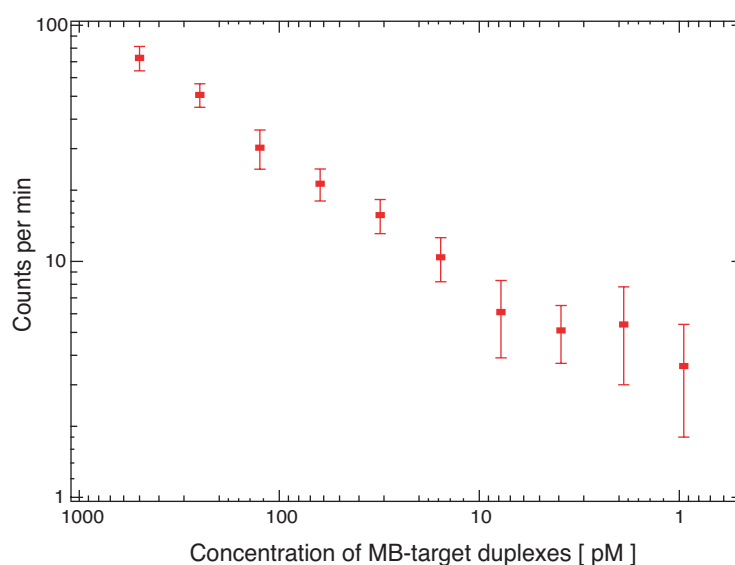
constant temperature of 26°C in a buffer solution. Individual fluorescence bursts produced by the oligonucleotide duplexes are clearly distinguishable from the background signal. The fluorescence bursts vary from 50 till over 1000 counts above the background signal. The amplitude of approximately 50 counts above the background signal correlates optimally with the count rate for one fluorescent burst resulting from one single molecule at a bin width of 100  $\mu$ sec [see chapters 4 and 5]. Peaks having an amplitude of less than 50 counts are highly suggestive for incomplete saturation of the donor fluorophore, improper fluorescence coupling into the light-guiding fiber core by the acceptor fluorophore or simply because the trespassing of the target molecule through the detection volume of the glass fiber sensor could not be completely acquired during one complete binning interval. The higher peaks accomplished due to the fact, that sometimes several single molecule events trespass the detection volume of the glass fiber sensor during the same binning interval, resulting in an addition of several single small bursts to a bigger one.

It should be emphasized that accurate detection of fluorescent labelled single-molecules in the present detection scheme can only be achieved by proper permanent stirring of the buffer solution and its components at a high rate [see chapter 4.3.1].

#### **6.4.2 Quantification of wavelength-shifting molecular beacons**

Fig. 7 shows the number of fluorescence bursts as function of the concentration of MB-perfect target duplexes after an initial hybridization time of ten minutes. It results from a dilution series of perfect-targets-wavelength-shifting-molecular-beacons-duplexes in a buffer solution. To assure a quick and proper hybridization of all the wavelength-shifting-molecular-beacons, the ratio of complementary targets to wavelength-shifting-molecular-beacons was 20:1 for the subsequent dilution

measurements. Dilution steps were performed by removing for each step half of the sample volume and substituting the missing volume with fresh buffer solution. In order to obtain a mean value, 10 separated measurements of 1 min duration were performed at each concentration. According to previously performed experiments [see chapter 4 and 5] we were also able to discriminate single fluorescence bursts resulting from hybridized wavelength-shifting molecular beacons to complementary targets from a strong normally distributed background.



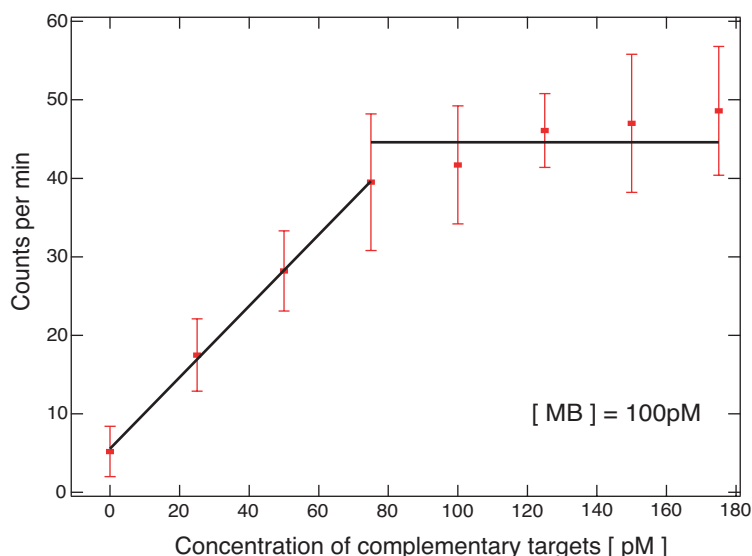
*Fig. 7: Dependence of the number of fluorescence bursts on the concentration of MB-target duplexes resulting from a dilution series. A linear dependence of the number of bursts as a function of the concentration is observed between 10 – 500 pM. Below 10 pM the curve starts to level off.*

#### **6.4.3 Quantification of complementary targets in relation to a fixed concentration of wavelength-shifting molecular beacons**

In order to demonstrate the possibility to quantify the concentration of targets a series of experiments have been performed in which the target concentrations has been increased continuously while the concentration of MBs was fixed at 100pM. While for small target concentrations the



number of detected fluorescence bursts increases linearly with the target concentration at higher target concentrations the number of bursts saturates. This is due to the fact that the maximum number of bursts is limited by the finite concentration of the wavelength-shifting MBs. As expected, concentration of perfect targets higher than the concentration of MBs does not result in a further increase of the number of fluorescence bursts detected per minute.



*Figure 8: A measurement series with increasing complementary target concentrations (0 - 180 pM) and fixed MB concentrations (100 pM) shows a plateau, since the maximum photon yield is determined by the amount of hybridized molecular beacons and is limited by the fixed concentration of MBs*

## 6.5 Conclusions

In this chapter the wavelength-shifting molecular beacons were successfully integrated into the system. It could be shown that wavelength-shifting molecular beacons in the absence of perfect targets do not produce significant additional signals that would interfere with the single molecule detection method. In fact, a solution containing MB in a concentration of up to 100 pM and no targets can hardly be

distinguished from a plain buffer solution. The experiments conducted proof that also with wavelength-shifting molecular beacons a linear dependency can be obtained when performing a dilution series. The linear dependency confirms that the sensor is also able to quantify free-floating wavelength-shifting molecular beacons-perfect-target-duplexes. In addition, it is also possible to quantify in only one minute the amount of molecular beacons that have hybridized to their target sequence. Furthermore this detection and quantification method can be conducted with a fixed concentration of molecular beacons, which simplifies the procedure, since the amount of MB needed does not have to be anticipated prior to an analysis.

We therefore successfully demonstrated the detection and quantification capabilities of a novel optical biosensor for unlabelled short oligonucleotide target sequences using wavelength-shifting molecular beacons in a liquid environment.

Future work will be directed towards the direct detection and quantification of target sequences relevant for medical diagnostics.

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## 7. Corollary

The here presented novel detection and quantification method highly qualifies for applications within the biomedical sectors. During the previous chapters the hard- and software has been systematically introduced and tested under various aspects. The algorithm presented in chapter 3 allows for proper discrimination of fluorescent bursts from a strong Poissonian background. Applying this detection method with the correct settings allows for adequate quantification of fluorescent-labelled molecules in solutions. The introduction of molecular beacons into the system poses no problems whatsoever, since the quenching efficiency of the BHQ2 is sufficient enough to allow the optical sensor to properly discriminate between the open and closed conformation of the molecular beacons. The efficiency of this quantification system ranges from a concentration of 100 fM to 1 aM, which easily surpasses any other oligonucleotide quantification method to date, especially when considering that no amplification such as PCR is needed. It further could be demonstrated that the sensor shows accurate sensitivity for detection of single molecules in the range of zeptomolar concentrations. As intended, chemical immobilization or adsorption procedures can be completely avoided. Since all the detection and quantification measurements were performed using an optical glass fiber, this method can be qualified as remote sensing and offers the potential of parallelization by using different fibers and/or laser wavelengths. It would then be possible to investigate several different targets within the same sample at the same time.

The additional parallel acquisition of different markers, such as life cell markers (e.g.  $\beta$ -actin, etc.) would allow to simultaneously quantify the measured genetic expression level in relation to a cellular reference value, since the cellular content can greatly vary within different tissue

types. The use of glass fibers also enables to separate reusable hardware components from the measuring fluid cell as claimed in chapter two. The use of integrated optics furthermore ensures the contingency for miniaturization, such as for interests in processing of a lab-on-a-chip. The method is fast. Quantification time rates were all within one minute, which is very rapid comparing today's existing tools in the market. Hence the detection system with its resolution capabilities also reserves the alternative of combination with microfluidic networks.

Considering the fact that up to the probe sequence of the molecular beacon the whole system with all its components can be retained unchanged regardless the target sequence of interest, this quantification method allows for an even broader spectrum of supplementary applications. Other possible fields of interests additional to the pharmaceutical and biomedical fields would be the farming sector, e.g. varmint detection, materials research, e.g. investigation of repellent surfaces and of course the military sector, e.g. the detection of ultralow concentrations of bioagents.

## **8. Outlook**

### ***8.1 Molecular Beacons and perfect targets in hemolyzed blood***

#### **8.1.2 Introduction**

Circulating RNA in plasma/serum is an emerging field for noninvasive molecular diagnosis [1-4]. The discoveries of tumor-derived RNA in the plasma/serum of cancer patients [5] and fetal derived RNA in the plasma of pregnant women [6] have opened up a new field for studying gene expression noninvasively. Problems for fluorescence-based detections methods, however, may arise if the absorbance and emission spectra of hemoglobin would crosstalk with the test system fluorochromes [5-7]. Initial experiments using perfect HER2/Neu target mRNA-molecules as targets that have been inserted into blood-lysate sample indicate that our biosensor might also work in blood containing corresponding molecular beacons. The molecular beacons were able to find their targets even in the presence of unspecific RNA and DNA within minutes. The autofluorescence of the hemoglobin was not interfering with the FRET signal of the molecular beacons.

#### **8.1.2 Experimental, results and discussion**

The here presented detection and quantification method has been conceived to operate in a clinical environment. The molecular beacons and perfect targets used in chapter 6 were used accordingly to their previously described specifications. The buffer solution was completely replaced by blood. The blood was extracted imminently before the experiment and stored in a citrate-containing syringe. The blood was then subsequently hemolyzed with a drop of conventional liquid soap.

The so hemolyzed blood sample was then placed in the fluid cell. After a brief phase of homogenization the fiber sensor was placed into the blood sample and the molecular beacons were added 14 seconds after the recording had been started (Fig. 1 (a)). A slight increase of the background mean count rate can be determined, due to imperfect quenching of the FRET pair (see chapter 7). The amplitude of the background however does practically not change. After 60 seconds the recording was restarted in order to maintain the data file down to a manageable size. After 70 seconds of the restarted recording the perfect targets were added (Fig. 1 (b), I). Although for reasons of display barely visible, a slight increase of the background can also be observed. Then the first hybridized beacons yield their fluorescent signal into the glass fiber. But it is only after almost 3 minutes that the majority of the molecular beacons starts to hybridize to their targets. This causes a massive increase of the background mean count rate and the amplitude as described in chapter 4 for the FRET target molecules. The single fluorescent bursts are clearly distinguishable from the background. In addition, some bursts consisting of collective signals can be perceived, as described in the previous chapters. The main concerns with this experiments were that the remains of corpuscular elements within a liquid solution would cause measuring artifacts by colliding with the glass-fiber sensor or that the autofluorescence of the hemoglobin molecules might interfere with the FRET signal of the molecular beacon.

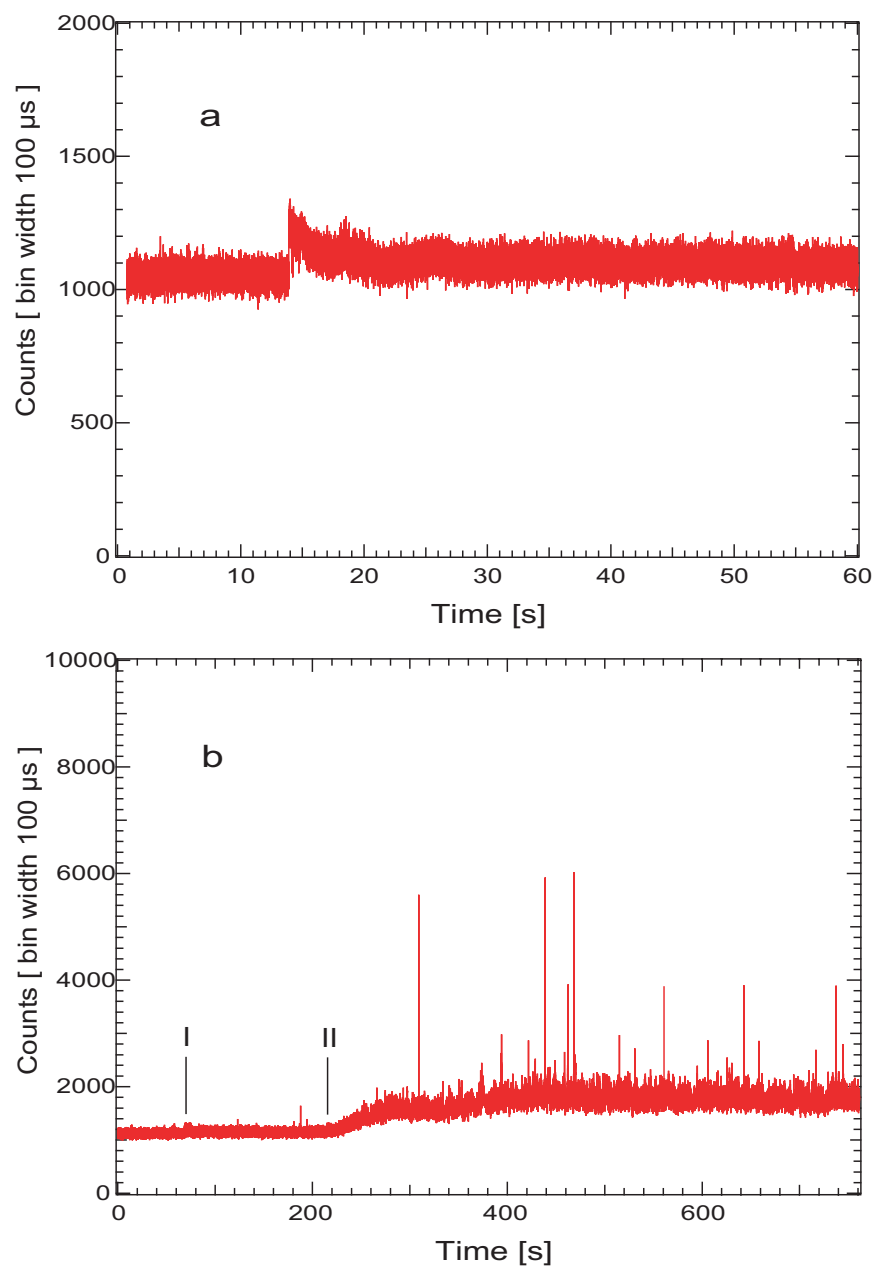


Figure 1(a) and (b): Contiguous acquisition of counts (bin width 100  $\mu$ sec) as a function of time for a demonstration of a real-time hybridization of molecular beacons to their perfect HER2/Neu target mRNA-molecules in hemolyzed blood. Insertion of molecular beacons (a) after 14 seconds of experiment time. Insertion of perfect mRNA targets after 70 seconds of the restarted experiment recording (b,I). Begin of hybridization (b,II).



### **8.1.3 Summary**

In summary, our preliminary experiments show, that our approach is capable of highly sensitive, highly accurate detection of perfect HER2 mRNA target molecules using molecular beacons within few minutes in hemolyzed blood. The main concern hereby was that remains of corpuscular elements within a liquid solution and the hemoglobin autofluorescence would cause measuring artifacts. The results however are quite encouraging, since neither the suspected autofluorescence from the hemoglobin nor any membran residuals provoked any disturbances of our measurements.

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## 9. Technical Drawings

In the following some of the plans produced by the workshop of the technical department of the Institute of Physics are presented. These blueprints show the design of the stirring device of the optical setup. In order to achieve rates of up to 25000 rpm, the demands for this device were very conservative. The stirring had to be performed at an adjustable rate, which was controlled by a specially designed experiment control software. The device mainly consists of a motor and a downstream tachometer. The also specially produced PMMA fluid cell could be fixed flush to the stirring device in order to avoid evaporation of the sample solution but had also to be operational in an open position to allow for proper diluting of solutions while the sample would be stirred constantly. Additionally the glass fiber sensor had to remain in an identical position for all measurements. Sample compartment is able to contain up to 1.5 ml of sample. However, most of the measurements were performed with an amount of 1 ml, if not precised otherwise.

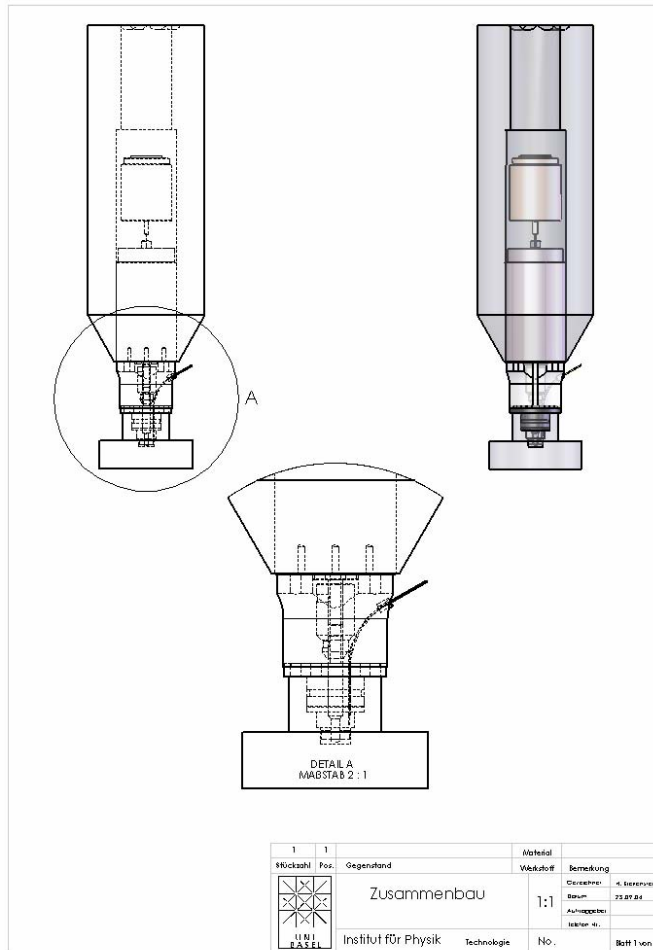
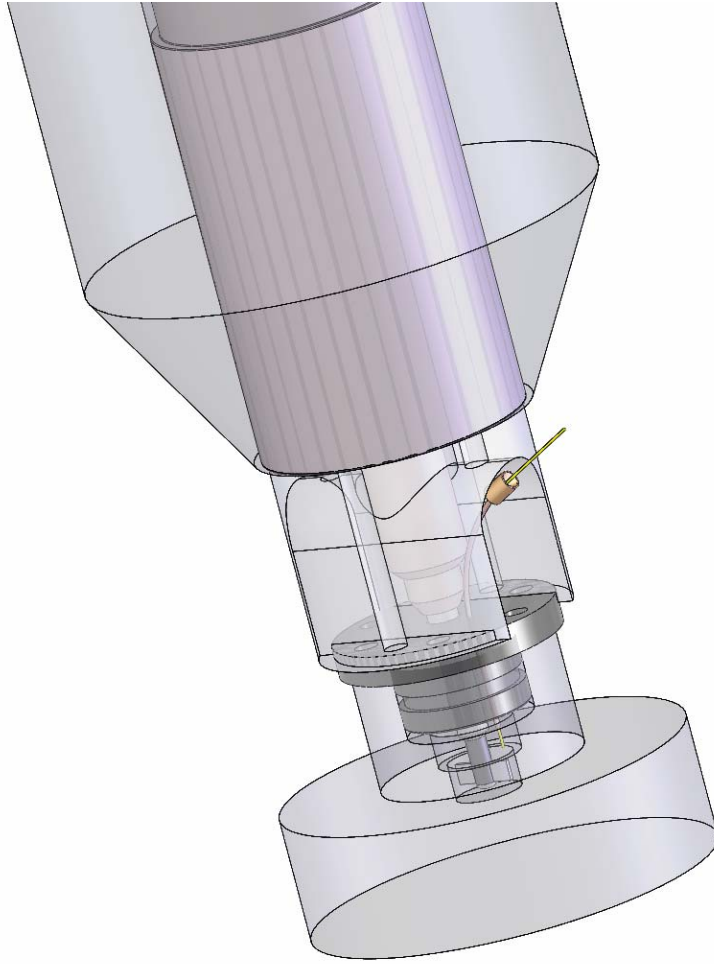
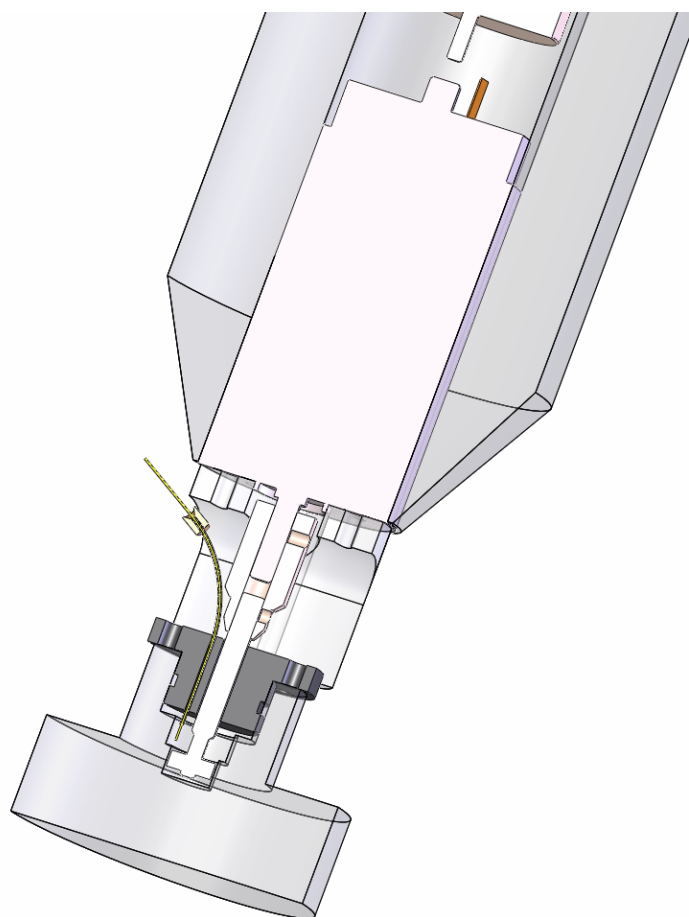


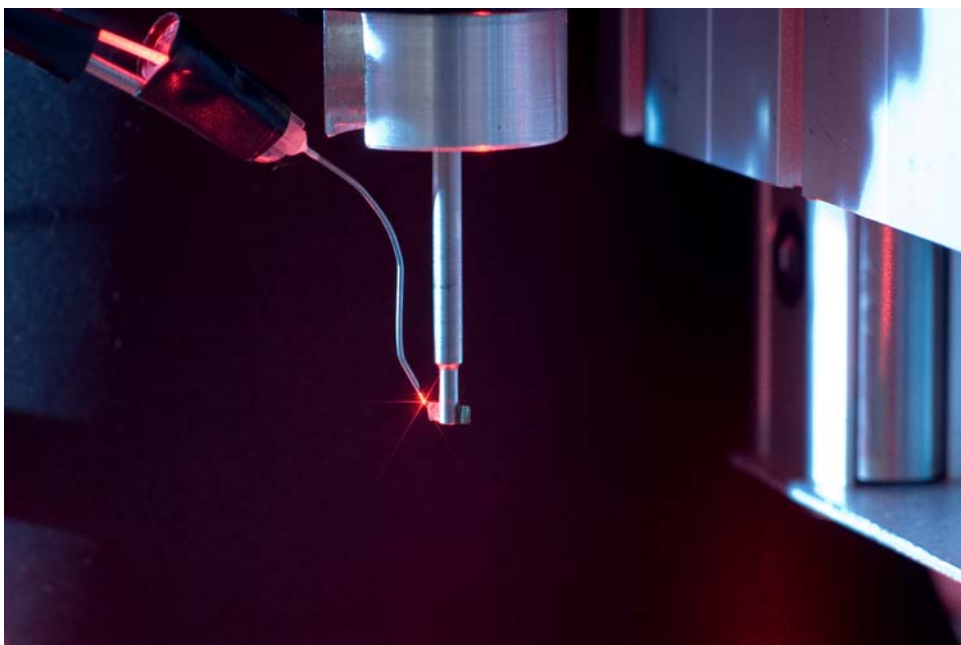
Figure 1 shows a blueprint of the main compartment of the stirring device with a magnification of the transition in the area of stirring axle. Note the insertion channel for the glass fiber sensor.



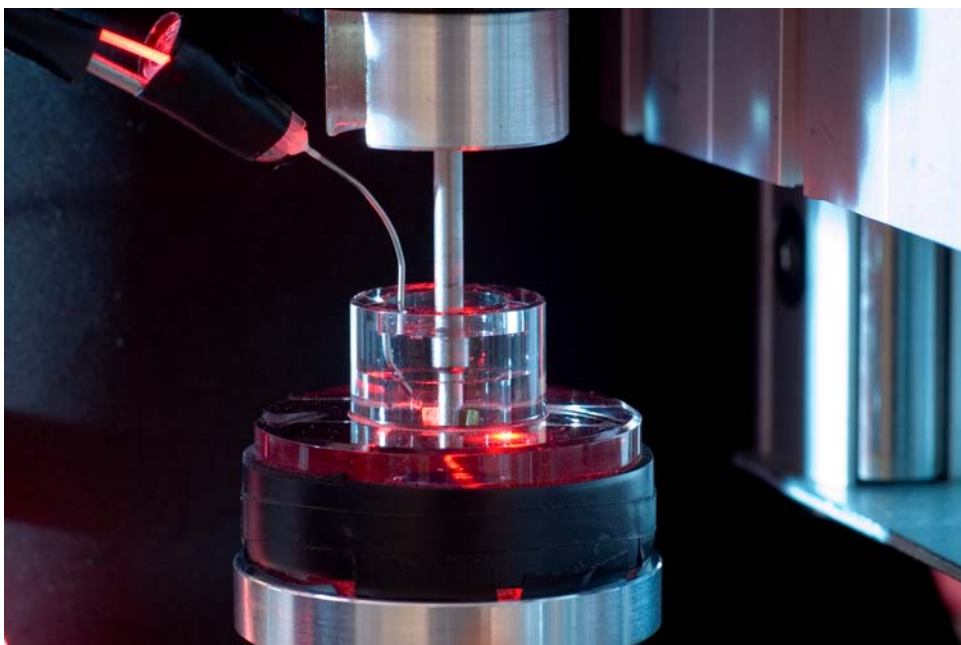
*Figure 2: This blueprint demonstrates the configuration for measurements under tight sealing of the plexi glass fluid cell. This setup configuration was chosen when sample evaporation had to be avoided, e.g. for the bleaching experiment (see chapter 5) or at least minimized, since the axle channel to the sample compartment was intentionally not perfectly sealed.*



*Figure 3: Cross section through the stirring device and plexi glass fluid cell. This Figure visualizes the relative position of the glass fiber sensor to the stirring blade. It also shows the sample compartment below the sealing cap (dark grey). Note the channel of the glass fiber (yellow).*



*Figure 4: Photograph of the stirring axle with blade and the glass fiber sensor. The sealing cap has been removed in order to allow for dilution experiments (see above). A 23 gauge hollow injection needle replaces the fiber channel. The excitation power at the end of the glass fiber is 2 mW. The plexi glass fluid cell is missing.*



*Figure 5: Photograph of the stirring axle with blade and the glass fiber sensor. The sealing cap has been removed in order to allow for dilution experiments (see above). A 23 gauge hollow injection needle replaces the fiber channel. The excitation power at the end of the glass fiber is 2 mW. The plexi glass fluid cell is placed in its apron. This configuration allows to remove and to add sample solution with a pipette under constant stirring. This configuration was applied for measurements that are described in chapters 6 and 7.*



## 10. Patent

### **(WO/2006/018706) SINGLE ANALYTE MOLECULE DETECTION BY FIBRE FLUORESCENCE PROBE**

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#### **Priority Data:**

60/602,332

18.08.2004

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**Title:** SINGLE ANALYTE MOLECULE DETECTION BY FIBRE FLUORESCENCE PROBE

**Abstract:**

An apparatus for single analyte molecule detection includes: a light source (20) for generating excitation light; a dichroic mirror (22) disposed on a first path of excitation light generated by the light source, wherein the mirror directs excitation light into a fiber aligner (30); an optical transducer coupled to the light source by the fiber aligner, the optical transducer comprising an optical waveguide (40) made of dielectric material having a first dielectrical index; a photon detector (70) disposed to receive fluorescent back radiation, wherein when a test solution having a second dielectric index lower than the first index is provided and comprises one or more target molecules, excitation light is transmitted by the waveguide and exits a waveguide tip disposed in the test solution so as to excite one or more target molecules; subsequently, the waveguide transmits back radiation along a second path to the photon detector that detects the transmitted back radiation.

**Designated States:**

AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

African Regional Intellectual Property Org. (ARIPO) (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW)

Eurasian Patent Organization (EAPO) (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM)

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**Patent**

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